THE REACTIONS BETWEEN BACTERIA AND ANIMAL TISSUES UNDER CONDITIONS OF ARTIFICIAL CULTIVATION.*

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PLATES 18 TO 20.

Realizing that in ordinary bacterial culture media as usually employed the conditions are far from ideal, and at best a lifeless inactive medium in contradistinction to the active living medium presented by the human or animal body, I was led, in the fall of 1913, to undertake a series of experiments with tissue cultures according to the now well known method of Harrison as modified by Burrows (1). These actively growing cell cultures were used as media on which to attempt cultivation of various pathogenic organisms, and some interesting and suggestive results were obtained which are reported here as opening up what seems to be a promising vista for more detailed observations along the same lines. In these growing cultures the cells are constantly splitting the protein bodies of the plasma to form amino-acids which even bacteria with no proteolytic enzymes can easily handle, and also here we have the opportunity, hitherto lacking, of observing from day to day, directly under the microscope, the interaction of bacteria and tissue cells in normal homogenous or heterogenous plasma, in plasma from immunized animals, and in artificial media which may be varied at will.

TECHNIQUE.

In most of the studies here reported chick embryo cultures were employed, especially heart tissue and splenic tissue from seven to fourteen day embryos. The former gives two indications of bacterial action, the rate and extent of cell multiplication and the rate and force of pulsations or the arrest of the same; while in splenic cultures connective tissue growth and leucocytic growth and migra-

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tion may be studied. Bacterial inoculations may be made on solidified cultures at varying intervals after making, into diluted plasma at or before the time of tissue planting, or as controls into plasma without tissue. With each series of cultures sterile controls of normal tissue should always be made, as conditions vary with each planting and results are valueless without comparisons under identical conditions of tissue, plasma, temperature, etc. In all inoculations, except those on solidified cultures twenty-four hours or more after planting, the natural bactericidal action of the plasma must be taken into consideration.

The organisms studied so far have been *Micrococcus aureus* and *Bacterium diphtheriticum,* both pathogenic to chickens and to man, a Hoffman type *Bacterium pseudodiphtheriticum* for comparative purposes, *Bacillus typhosus,* pathogenic to man but not to chickens, *Bacillus coli verus,* usually non-pathogenic and normal to both man and chickens, and *Bacillus prodigiosus,* a non-pathogenic air organism. The diphtheria and typhoid organisms gave the most interesting and characteristic results, but the actions of all organisms used were instructive when comparisons were made. Cultures were made in series and observed daily, and specimens were fixed and stained for permanent preparations at regular intervals. With diphtheria, observations on the action of toxin and antitoxin on tissue cultures were also made. In judging results it must be remembered that one is dealing with very minute fragments of tissue, and to approach normal conditions, bacterial inoculations should be very light. In most series two classes of cultures were made, small cover-glass hanging block preparations with one or two drops of diluted plasma and one to four tissue fragments, and larger cultures on the under surface of lids of glass boxes, the culture containing five to ten drops of diluted plasma and numerous tissue fragments. Where plasma inoculations are made the best plan is to inoculate the diluting fluid, sterile water, or Ringer's saline solution, before adding it to the plasma, and if light inoculations are desired two or more dilution tubes in series should be used, as in plating on agar. A dilution of one part water or saline to two parts plasma gives good results for routine work. In all instances agar slants were made from inoculated plasma tubes to control the inoculations.

BACILLUS PRODIGIOSUS AND MICROCOCCUS AUREUS.

As the two extremes of action, *Bacillus prodigiosus* and *Micrococcus aureus* may be taken. *Prodigiosus* failed to develop colonies in chicken plasma without tissue, and in most cases also in the presence of tissue. Thiele and Embledon (2) claim that when inoculated from a hypertonic medium *prodigiosus* becomes pathogenic owing to the protective action of the saline preventing early bacteriolysis, and in confirmation of this fact it was found that when *prodigiosus* was grown in hypertonic bouillon (3 per cent. sodium chloride) it would invariably develop a few colonies in tissue cultures. In contrast with *prodigiosus, Micrococcus aureus* invariably grew very freely and vigorously under all conditions in such cultures with or without tissue, and with all tissues used. With most tissues, while the *aureus* colonies were abundant throughout the culture, they were larger and more numerous near the tissue fragments and entirely prevented or very much inhibited tissue growth. With splenic cultures, however, and to a less degree with embryonal liver tissue cultures, this was not the case. If inoculations were heavy the colonies clustering around such tissue fragments were fewer and much smaller, while with moderate and light inoculations these tissues were surrounded with clear bacteria-free halos almost coëxtensive with the areas of migration of the lymphatic cell elements. This contrast between heart and splenic tissue action is well shown in figure 1 where fragments of both tissues have been planted in the same infected culture.

BACILLUS TYPHOSUS AND BACILLUS COLI VERUS.

Bacillus typhosus and *Bacillus coli verus,* closely related to each other in morphology and cultural characteristics, showed marked contrasts in tissue cultures, more sharply marked than in ordinary laboratory differential tests for the same. *Bacillus typhosus* never in a single instance in ninety tests showed any development in plasma cultures, with or without tissue, when inoculated from isotonic or from hypertonic media. That this was due to strong bactericidal action of the chicken plasma for *Bacillus typhosus* was shown by the following results: when freshly solidified cultures or six hour old

cultures were inoculated with this organism, by means of a platinum needle dipped in a heavy suspension, no bacterial growth occurred; but if the cultures were first incubated for twenty-four hours and then inoculated there was usually a light spreading growth; and if incubated for forty-eight hours before inoculation there was always a heavy growth, the incubation having destroyed the complement at least, if not the amboceptor, in the plasma which had prevented bacterial development. That the bacteria were actually killed was shown by lack of growth on agar slants from such cultures after incubation. *Typhosus* growth developing on such incubated cultures did not interfere with tissue cell growth or pulsation of heart tissue for at least forty-eight hours, or until the tissue cells were heavily coated with the encroaching bacteria, which had a tendency, in old cultures especially, to cling to the new cells like vines to a decaying tree, giving the picture in stained cultures referred to by the author as "bacterial trees," as seen in figure 2. This same appearance was seen with one other organism, a spore-forming rod of the *subtilis* type developing as an accidental contamination. In one instance only was it possible to overcome the bactericidal action of fresh plasma on *Bacillus typhosus;* here the moist tissue fragments were covered with a heavy suspension of active typhoid organisms several minutes before the plasma was added, thus giving an extremely heavy dose of organisms in proportion to plasma. In contrast with the failure of typhoid bacilli to develop in tissue cultures, *Bacillus coli verus* seems to be entirely uninfluenced by any bactericidal properties of chicken plasma and to grow freely in vigorous colonies generally scattered throughout the plasma and, if the inoculation is heavy, to have a decidedly inhibitory action on tissue cell growth. Growth was also vigorous in clear plasma without tissue fragments. The clear halos seen around splenic fragments with *Micrococcus aureus* were absent with *coli verus,* the colonies developing up to and on the splenic tissue as vigorously as elsewhere.

BACILLUS DIPHTHERITICUM AND BACILLUS PSEUDO-DIPHTHERITICUM.

The most interesting results of bacterial infections of tissue cultures were those obtained with *Bacterium diphtheriticum* in contrast

to those with *Bacterium pseudodiphtheriticum*. With heavy inoculations both organisms develop freely, with or without tissue fragments, but when tissue is present by far the most vigorous colonies are seen immediately surrounding the tissue fragments. With diphtheria bacilli in such cases the tissue makes no growth and heart fragments do not pulsate, but with pseudodiphtheria bacilli, even with heavy inoculations, there is some new cell growth, and heart fragments may at times pulsate when laden with colonies. With moderate or light infections, however, there is a far more marked contrast. Pseudodiphtheria bacilli in light inoculations only occasionally develop and then only an individual colony survives here and there through the plasma, this happening more often in the smaller cover-glass preparations than in the box cultures. If inoculated from hypertonic bouillon, however, it usually developed more freely, as did also *prodigiosus*. Diphtheria bacilli in light or moderate inoculations showed a striking and peculiar development, the colonies appearing in numbers clustered around the individual tissue fragments, as in figure 3, and never in the clear plasma away from tissue, or in plasma controls with no tissue. In box cultures containing numerous tissue fragments each fragment resembled a planet with numerous satellite colonies around it. With splenic tissue the colonies clustered around the outer limits of the zone of lymphatic cell migration, leaving a clear zone next to the original tissue which resembled a sun, the center of a microscopic solar system (figure 4). The bacilli, ordinarily destroyed, if not in too great numbers, by the bactericidal action of the plasma, seem to be afforded some protection or stimulation by some cell secretion or product of cell metabolism which enables them to overcome the bactericidal influence of plasma. This fact may explain why clinically diphtheria is a local infection and rarely, if ever, a bacteremia, except in pre-agonal states where the plasma has lost its bactericidal properties. The clear, bacteria-free halo was lacking around a culture of splenic tissue from a seven day chick embryo, but at this time the spleen has little, if any, lymphatic elements and shows a pure connective tissue type of growth. That the failure of bacilli to grow in plasma without tissue was due to a bacteriolysis was shown by comparing the results of cultures made from the same batch of plasma one

and four days after its inoculation with diphtheria organisms, in the former case there being numerous colonies clustered around and through the heart tissue fragments, while in the latter there was only an occasional colony developed. As a general rule, heart cultures infected with diphtheria bacilli pulsated less vigorously and ceased pulsation much earlier than sterile controls in the same series, and when solidified cultures, after incubation, were inoculated pulsation usually ceased within twenty-four hours after the bacterial growth had reached the tissue fragment. With moderately heavy plasma inoculations the tissues at first showed very slight evidence of new growth, as in figure 3, but after twenty-four to forty-eight hours, when the cells seem to have produced antitoxin enough to overcome the toxic action of the colonies, some new cell growth begins to take place. Previous addition of diphtheria antitoxin to the plasma did not influence bacterial growth, but it did prevent deleterious action of the bacilli on the tissue cells. An interesting confirmation of the close relation between true diphtheria bacilli and pseudodiphtheria bacilli was seen when pseudodiphtheria inoculations were made into plasma containing diphtheria toxin. In these cases pseudodiphtheria organisms behaved just as did true diphtheria in ordinary cultures, the colonies developing regularly and always clustering around the tissue fragments.

SPLENIC EXTRACT.

To endeavor to determine if the clear halos around splenic cultures seen in *aureus* and *diphtheriæ* inoculations were due entirely to phagocytosis of bacteria by lymphatic cells, extracts of embryonal splenic pulp were prepared according to the method used by Carrel (3) in his work on growth stimulation, and these extracts, after storage for ten days or more on ice, were dropped on solidifying cultures of tissue with bacteria. In every instance the area covered by the splenic extract remained free, or nearly free, from colonies, while the remainder of the culture was filled with them. Embryonal liver, also rich in lymphatic elements, had a similar action to that of spleen, though not to so marked a degree.

DIPHTHERIA TOXIN.

In addition to the above experiments with bacterial inoculations a series of experiments was carried out to show the action of diphtheria toxin and antitoxin on cell growth. The toxin was added in varying amounts to the plasma before culturing, the dosage usually being calculated for the amount of plasma, not of tissue which was subject to more variation, in proportion to toxin, from the standard of the L. + dose of toxin employed for a 250 gram guinea pig; 0.5 of a cubic centimeter of plasma, the amount used for one box culture, containing 1/500 or N/500 of the L. + dose. In general, chick tissues seem to have a much higher resistance to toxin than do guinea pigs, for one L. + dose had little or no effect on cultures. Larger doses, however, had an increasing inhibitory action and if sufficiently large the growth and pulsation of heart tissue were prevented, as were growth and migration of lymphatic elements from splenic tissue. Moderately heavy doses, not sufficient to kill tissue, arrested growth for twenty-four to forty-eight hours, after which time some new cells began to develop. Growth of nervous tissue was arrested by decidedly smaller doses than was that of any other tissues. New cells developing from cultures with large doses of toxin were few in number and showed early advanced degeneration, heavy accumulation of fat droplets, blunted processes, and dense inactive or fragmented nuclei (figure 5). Cultures from the same series with the same dose of toxin but with also a corresponding dose of antitoxin showed fairly vigorous growth with many normal or nearly normal cells (figure 6).

SUMMARY.

This report gives an outline of the results of observations on over 1,100 tissue cultures made during the fall, winter, and spring of 1913 to 1914. The work has been resumed in the fall of 1914 and will be continued along the same and allied lines, confirming the above results with other strains of the same organisms and with other bacteria and bacterial products. Tests should be made with tissue and plasma from other animals, and the known pathogenicity of the organism for the animal and tissue used should always be

borne in mind. We hope to be able to grow on these cultures some of the more strictly parasitic bacteria not developing on ordinary media, as other strict parasites have been grown by other workers, *viz.,* poliomyelitis virus by Levaditi (4), vaccinia by Steinhardt, Israeli, and Lambert (5), and rabies by Moon (6). By comparative studies with various types of cells and various natural and artificial media, clearer ideas as to the exact part of cell plasma in antibody production, by elaborating on the methods of Carrel and Ingebrigtsen (7), Lüdke (8), Przygode (9), and others, should be possible.

After this work was begun a reference was found to the use of some pathogenic bacteria in tissue cultures by Pheiler and Lentz (10), but no publication of the results of these observations has been observed.

The results here reported may be summarized as follows:

Bactericidal Action of Chicken Plasma.—On *Bacillus typhosus,* very strong—never grows in plasma alone; on *Bacillus prodigiosus,* very strong—never grows in plasma alone; on *Bacterium pseudodiphtheriticum,* strong—slight growth in cover-glass preparations; on *Bacterium diphtheriticum,* moderately strong; on *Bacillus coli verus,* slight; on *Micrococcus aureus,* very slight or none.

A few pseudodiphtheria bacilli and more diphtheria bacilli survived in plasma stored in the cold for four days. The presence of growing tissue overcomes the bactericidal influence of plasma on diphtheria bacilli and in some instances on pseudodiphtheria bacilli.

Bacterium diphtheriticum grows in plasma without tissue only if inoculations are very heavy; and very heavy inoculations of all organisms will probably overcome the bactericidal action of plasma, as it is undoubtedly a quantitative reaction. The bactericidal influence of plasma is overcome by exposure to incubator temperature for twenty-four to forty-eight hours. *Bacterium diphtheriticum* in light or moderate inoculations grows in tissue cultures only in clusters around the tissue fragments, and never in plasma away from tissue. The growth of this organism has a decided inhibitory influence on tissue activity and growth, especially marked with nervous tissue, but this action may be overcome by the addition of antitoxin to the plasma. Cultures inhibited by diphtheria growth

have a tendency to resume growth later, probably due to antitoxin production.

Bacterium pseudodiphtheriticum is distinctly less active in tissue cultures than is *Bacterium diphtheriticum* and never develops in plasma without tissue. The presence of diphtheria toxin in tissue cultures causes this organism to behave as does *Bacterium diphtheriticum.* Without toxin it has little or no direct influence on tissue growth except in massive doses.

Bacillus prodigiosus fails to develop, as a rule, in tissue cultures except where inoculated from hypertonic media, and then it has no decided influence on tissue growth.

Micrococcus aureus grows freely in these cultures with or without tissue, and inhibits tissue growth markedly, except as noted with splenic tissue.

Bacillus coli verus always grows freely with or without tissue fragments and is uninfluenced by splenic tissue growth. In heavy inoculations it lessens tissue growth.

Bacillus typhosus, except with extremely heavy inoculations, fails absolutely to grow in these cultures with or without plasma, unless the bactericidal action of the plasma has been destroyed by incubation. When this is the case it develops freely with especial affinity for the tissue cells either for support or nourishment. It appears to have no toxic action on the tissue cells. Note the sharp differentiation between typhoid and *coli verus* organisms.

Diphtheria toxin has a quantitatively inhibiting action on all tissue growth and on heart tissue pulsations, the action being greatest on nervous tissue and least on heart tissue growth. Tissues affected by toxin tend to recovery if not killed. Antitoxin counteracts the action of toxin.

Splenic tissue has little or no effect on the growth of *Bacillus coli verus,* but has a decided bactericidal action on *Bacterium diphtheriticum* and *Micrococcus aureus,* probably due to lymphatic cells and cell products, as seen by the area of cell migration coinciding with the bacteria-free area, by the similar action of splenic extract on cultures, and by the failure of such action in cultures of very early splenic tissue showing no lymphatic cells.

CONCLUSIONS.

We have in tissue cultures *in vitro* a promising addition to our methods of bacteriological study. The reaction of bacteria to tissue cultures would seem to be more or less parallel to the pathogenicity of the organism for the animal supplying the tissue.

This method promises a reliable means of differentiation between some pathogenic and non-pathogenic organisms of the same species. It will probably shed more light on the protective action of tissue cells and cell products against bacterial action, as is seen with splenic cultures.

It will probably explain more clearly the action of pathogenic bacteria and the definite relation they bear to the tissues involved and to the blood plasma, as is seen from the peculiar behavior of *Bacterium diphtheriticum* in tissue cultures.

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6. Moon, V. H., *Jour. Infect. Dis.*, 1913, xiii, 232.
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10. Pheiler, W., and Lentz, W., *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1913, lxviii, 122.

EXPLANATION OF PLATES.

PLATE 18.

FIG. 1. Twenty-four hour culture from twelve day chick embryo heart and splenic tissue in plasma inoculated with *Micrococcus aureus* before planting. Notice the limited tissue growth and general distribution of bacterial colonies, except in the clear halo around the splenic fragment. Magnified 30 diameters. H = heart fragment. S = splenic fragment. B = bacterial colonies.

FIG. 2. Seven day culture from eleven day chick embryo heart tissue in plasma. This culture was inoculated with *Bacillus typhosus* on the fifth day of incubation and shows new cell growth heavily invested with bacilli ("bacillary trees") with few or no bacilli between the cells. Magnified 220 diameters. H = heart fragment. C = new cell growth. T = bacteria-laden cells.

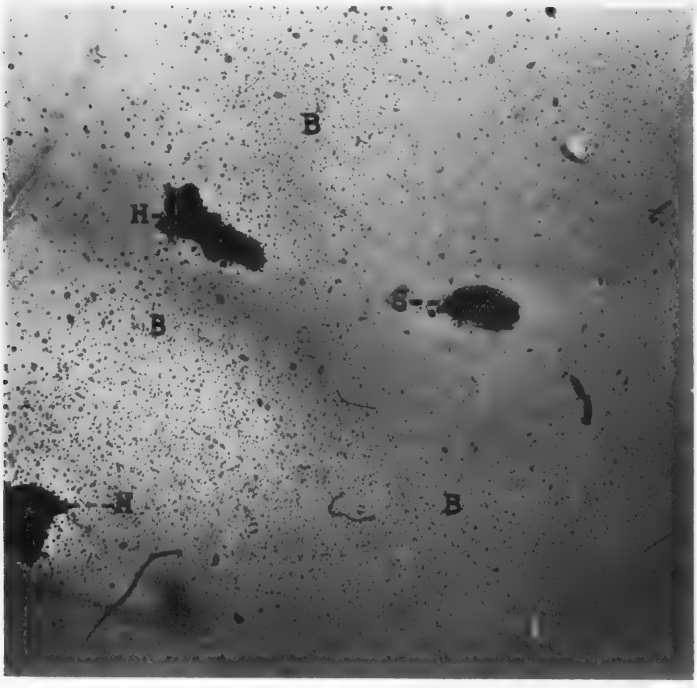

FIG. 1.

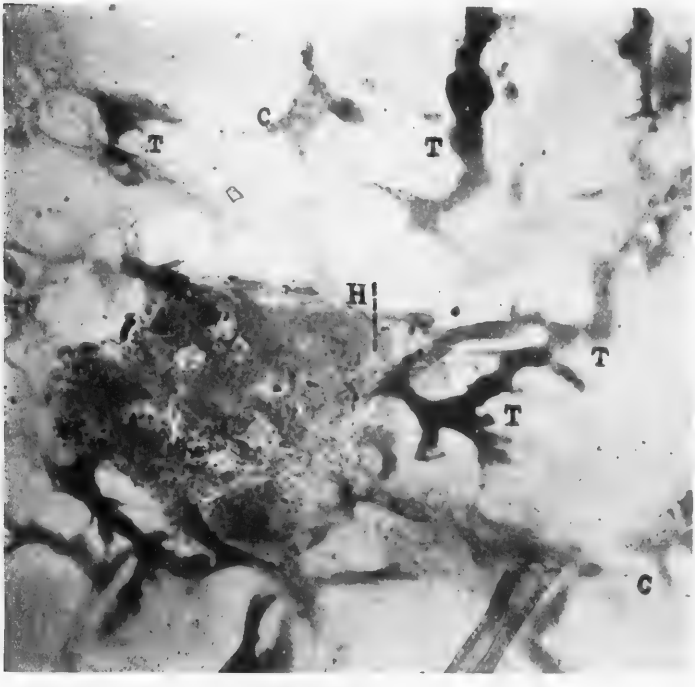

FIG. 2.

(Smyth: Reactions between Bacteria and Animal Tissues.)

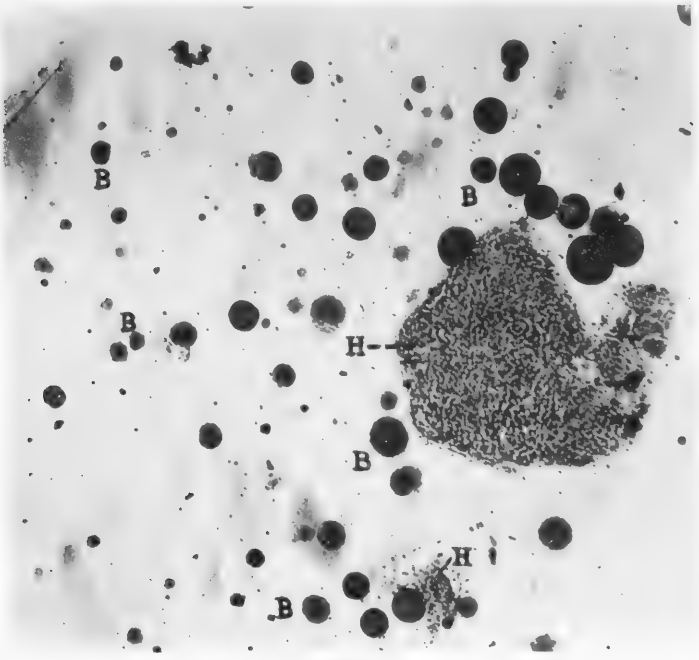

FIG. 3.

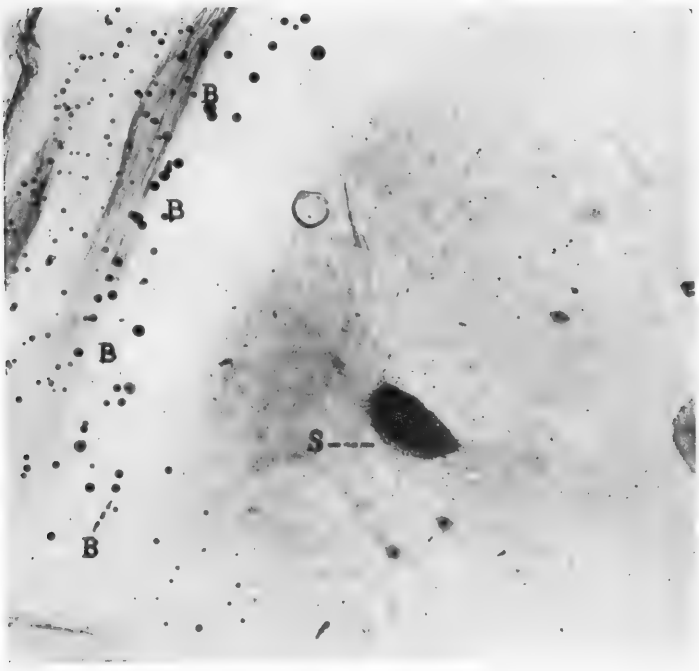

FIG. 4.

(Smyth: Reactions between Bacteria and Animal Tissues.)

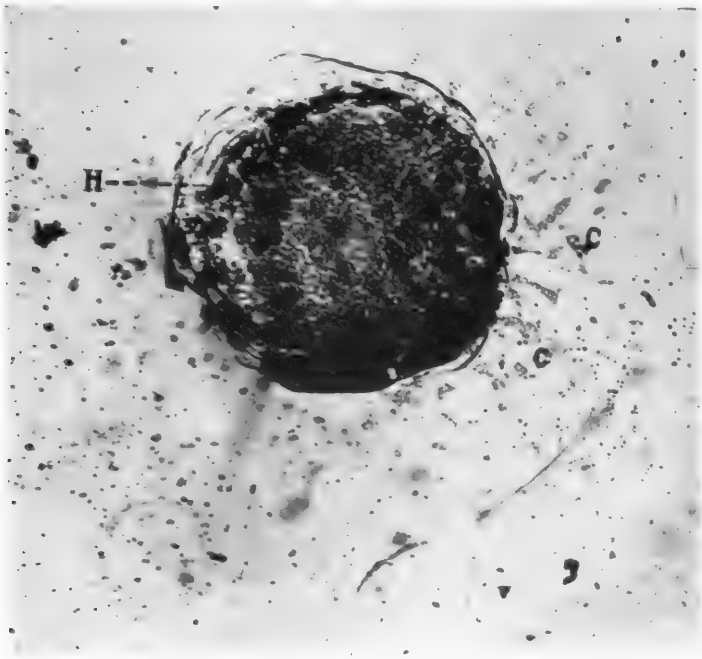

FIG. 5.

FIG. 6.

(Smyth: Reactions between Bacteria and Animal Tissues.)

PLATE 19.

FIG. 3. Twenty-four day culture from nine day chick embryo heart tissue in plasma inoculated with *Bacterium diphtheriticum* before planting. Notice the diphtheria colonies clustered around the tissue, with very little new tissue cell growth. Magnified 53 diameters. H = heart fragment. B = bacterial colonies.

FIG. 4. Two day culture from thirteen day chick embryo splenic tissue in plasma inoculated with *Bacterium diphtheriticum* before planting. Notice the large halo around the splenic tissue free from diphtheria colonies. The magnification is too small to show new tissue cells in this area. Magnified 12 diameters. S = splenic fragment. B = bacterial colonies.

PLATE 20.

FIG. 5. Four day culture from seven day chick embryo heart tissue in plasma plus diphtheria toxin. Note the limited growth of highly inoculated cells. With higher magnification these cells show degenerated fragmenting nuclei. Magnified 53 diameters. H = heart fragment. C = new cell growth.

FIG. 6. Four day culture from seven day chick embryo heart tissue in plasma plus diphtheria toxin and antitoxin. (Same toxin dosage as in figure 5.) Note the nearly normal new growth of healthy cells. Magnified 53 diameters. H = heart fragment. C = new cell growth.

VARIETIES OF PNEUMOCOCCUS AND THEIR RELATION TO LOBAR PNEUMONIA.*

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In a previous paper Dochez and Gillespie[1] have shown that pneumococci derived from lobar pneumonia may be divided according to their immunological reactions into certain well defined groups. There developed from this study the fact that pneumococci fall into two general groups. The larger of these, consisting of about 80 per cent. of the strains encountered, can be further subdivided into smaller groups. The latter have been arbitrarily numbered groups I, II, and III, and it has been found that a single member of either group I or group II is characterized by the possession of immunity reactions identical with those of other strains of the homologous group. Group III consists of the type known as *Pneumococcus mucosus,* and the first classification of organisms into this group depended upon differences of morphology and cultural reaction. A subsequent study by Hanes[2] has shown that members of the *mucosus* group manifest cross agglutination, so that it has been possible to relate further the members of this group by means of at least one immunological test. The immunity reactions of these three groups apparently do not change on artificial cultivation or on animal passage.

The smaller of the two main groups, which has been named group IV, is peculiar in that it seems to consist of a series of independent varieties which do not cross in their immune reactions with members of groups I, II, or III, or with each other. This group represents about 20 per cent. of the strains obtained from cases of lobar pneumonia, and this frequency seems to be fairly constant from year to year.

In view of our ability to recognize by means of specific reactions

* Received for publication, November 27, 1914.

[1] Dochez, A. R., and Gillespie, L. J., *Jour. Am. Med. Assn.,* 1913, lxi, 727.

[2] Hanes, F. M., *Jour. Exper. Med.,* 1914, xix, 38.

definite types of disease-producing pneumococci, we have deemed it important, in addition to confirming the constancy of these types, to study also the character of the pneumococci present in the normal human mouth, and on the mucous membranes of patients recovered from pneumonia, and to determine whether these strains differ in any way from those encountered during disease. If the type of organism that lives as a saprophyte on normal mucous membranes could be differentiated by certain fundamental and constant reactions from the ordinary disease-producing type, a reconstruction of our ideas concerning the epidemiology of lobar pneumonia would become necessary. Owing to the apparent lack of contact infection in pneumonia, investigators have been led to assume that the disease is due in most instances to infection with the pneumococcus that has been previously harbored in the mouth of the infected individual. If the strains present in normal mouths should show constant differences from the types found during actual disease, and if the latter types should be absent from the normal mouth flora, the likelihood would become great that infection in pneumonia takes place either by contact with infected individuals or apparently healthy carriers, however occult the steps which lead to infection may be.

During the past year we have been able to make a more extended study of the serological relationships of strains of pneumococci obtained from lobar pneumonia. The methods of classification have been the same as those previously employed; namely, agglutination and specific protection of animals against infection. Up to the present time no new group relationships have been discovered, and the recurrence of the types previously described has been constant.

In table I is shown the relative frequency of occurrence of organisms of the different types during the year 1912–13.

TABLE I.

	No.	Per cent.
Group I	35	47
Group II	13	18
Group III (*mucosus*)	10	13
Group IV (heterogeneous)	16	22
Total typical	58	78
Total heterogeneous	16	22
Total	74	

Table II shows a like classification of the strains obtained during 1913–14.

TABLE II.

	No.	Per cent.
Group I	21	30
Group II	28	39
Group III (*mucosus*)	6	8
Group IV (heterogeneous)	16	23
Total typical	55	77
Total heterogeneous	16	23
Total	71	

Tables I and II show the constancy with which the four groups described occur in two successive years. The percentage incidence of infection with members of the typical groups and with strains belonging to the heterogeneous group IV has been practically the same in both years. In 1912–13 the dominant type was group I. During 1913–14 the incidence of group I has diminished somewhat and there has been a corresponding rise in the number of infections with organisms belonging to group II.

The study of the various groups has brought out the interesting fact that the four types described differ in their degree of virulence for human beings. It has been impossible for us to obtain absolute figures of the mortality due to the different groups, because most of the individuals infected with groups I and II have been treated with specific antisera. In spite of the alteration in mortality brought about by this method of treatment, it has become clear that certain of the groups are more likely to cause a fatal infection than others. In general the severest forms of pneumonia result from infection with organisms belonging to groups II and III. The average virulence of group I seems to be somewhat lower, and, though infection with this type is usually severe in character, the mortality is definitely lower than in corresponding individuals infected with organisms belonging to groups II and III. The lowest grade of virulence is manifested by organisms of the heterogeneous group IV. Although infection with this group may run a severe course, it is unusual for it to terminate fatally.

These studies demonstrate the constancy with which the groups of pneumococcus described occur in New York City, and have now

been carried on for a sufficiently long period of time to render it unlikely that new groups closely related by immunity reactions will be encountered. In addition to these observations, investigations carried on by Dr. I. C. Walker,[3] in the Peter Bent Brigham Hospital, Boston, and by Dr. Paul Lewis,[4] in the Pennsylvania Hospital, Philadelphia, have shown that in these two cities the same groups of pneumococci are concerned in the causation of lobar pneumonia. Recently, by the use of immune sera prepared in this country, Professor Neufeld[5] has demonstrated the existence in Germany of groups of pneumococci having immune reactions identical with organisms belonging to our groups I and II. Previous study by Schottmüller[6] has already demonstrated the association of *Pneumococcus mucosus* with lobar pneumonia in Germany. Through the kindness of the South African Institute for Medical Research we have recently been able to test strains of pneumococcus isolated from cases of lobar pneumonia among natives in the Rand. The interesting observation has been made that even in this remote region of the world typical representatives of our groups I, II, and III are the causative agents in the production of lobar pneumonia. Lister[7] has described five groups of pneumococcus among the strains studied by him in South Africa. Three of these groups are identical with the groups met with in North America and in Germany. The other two groups, one of which appears to be dominant in South Africa, have not as yet been found in the cases of pneumonia studied by the writers. The possible significance of these two new races of pneumococcus will be discussed later in this paper.

STUDY OF STRAINS OF PNEUMOCOCCUS ISOLATED FROM THE MOUTHS OF NORMAL INDIVIDUALS.

Although there are in the literature instances of the apparent contagiousness of lobar pneumonia, it has been commonly assumed that most cases of the disease represent probable infection with a pneu-

[3] Personal communication.

[4] Personal communication.

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[7] Lister, F. S., *South African Institute for Medical Research* [*Publications*], Dec. 22, 1913.

mococcus dwelling during health on the buccal mucous membrane of normal human beings. Owing to a sudden accession of virulence of the pneumococcus, or unusual depression of resistance of the individual, this organism is supposed to be able to penetrate the lungs and set up disease. In view of the constant relationship of certain definite groups of pneumococci to lobar pneumonia, an opportunity has been afforded to test the validity of this assumption.

Isolation of pneumococcus from the sputum of a large proportion of healthy individuals has been fairly easy. Care was taken to avoid persons in direct contact with cases of lobar pneumonia, and the organisms were obtained at a season when the incidence of the disease is at a low ebb. Organisms which did not fulfill the requirements necessary for identification of the pneumococcus were discarded unless retained for special reasons. Owing to the large number of tests necessary for sufficient comparison of the organisms, the study was limited to fifteen different strains obtained from separate sources. One of these belonged to the group of *Streptococcus viridans* and was carried along with the rest for purposes of comparison, and in order to see if any important change in character occurred in this organism during the period of manipulation. All organisms were first tested for agglutination and protection with sera developed from immunization of animals with standard members of pneumococcus groups I and II. Subsequently rabbits were immunized with the strains from normal sputum, and cross relationships between these strains were tested. The general result of the investigation has been to demonstrate that strains of pneumococcus isolated from normal sputum do not belong to any of the fixed groups of pneumococcus, *i. e.*, groups I, II, or III, but resemble, as far as we can determine, the heterogeneous group IV, which has been previously described. Of the fifteen strains examined, in thirteen the morphology was that of a typical lance-shaped diplococcus. Of the latter, eleven strains were typical pneumococci in every way, showing the characteristic encapsulation, bile solubility, cultural and fermentative reactions, and two showed certain variations such as the absence of capsules even after repeated observation under suitable conditions and a tendency to precipitate in broth media. Both of these strains were bile-soluble; one fermented, and the other failed

to ferment inulin. Of the two strains showing morphological variations one was *Streptococcus viridans,* and the other a bile-soluble, inulin-fermenting organism which morphologically and culturally resembled *Pneumococcus mucosus.* It produced a characteristic sticky exudate in the peritoneal cavity of infected animals, but repeated attempts failed to demonstrate clearly defined capsules. The virulence of the different strains varied from a relatively high degree to practical non-pathogenicity for white mice. On the whole, the virulence of the most typical organisms was greater than that of those presenting variations. Of the fifteen strains eleven were typical pneumococci, three were closely enough related to be included in the pneumococcus group, and one was *Streptococcus viridans.* The serum reactions of all the strains were tested in the manner described in a previous paper on the serological reactions of *Pneumococcus.* A study was first made of the power of immune sera derived from stock cultures of groups I and II to agglutinate and to protect against infection with the sputum strains. In preparing the agglutination reactions serum and twenty-four hour broth cultures were mixed in equal quantities in the tubes. Readings were made after two hours at 37° C. and twenty-four hours on ice. The results of the agglutination tests are shown in table III.

TABLE III.

Agglutination of Sputum Strains with Stock Sera of Groups I and II.

Culture of pneumococcus.	1		2		3		4		5		6		7		8		9		10		11		12		13		14		15	
Hours.	2	24	2	24	2	24	2	24	2	24	2	24	2	24	2	24	2	24	2	24	2	24	2	24	2	24	2	24	2	24
Group I.	o	o	+	++	o	o	o	o	o	+	o	o	o	+	o	+	o	+	o	o	o	o	o	o	o	o	o	+	o	±
Group II	o	o	o	o	o	o	o	o	o	+	o	o	o	o	o	o	o	o	o	o	o	o	o	o	+	++	o	+	o	o
Normal horse serum.	o	o	o	+	o	o	o	o	o	+	o	o	o	±	o	±	o	o	o	o	o	o	o	o	o	o	o	+	o	o

Of the fifteen strains presented in this table, only two showed agglutination with the type sera at the end of two hours. In all, eight strains showed variable degrees of agglutination at the end of twenty-four hours. Of these eight strains, five agglutinated in more than one of the sera employed in the tests, and in most instances the heterologous agglutination occurred in normal horse serum, thus showing a tendency of the sputum strains to undergo

spontaneous agglutination. Such a variation practically never occurs in strains of pneumococcus isolated from lobar pneumonia, all of which either show specific agglutination or, when this does not occur, remain uniformly suspended for twenty-four hours. Of the fifteen cultures tested, No. 13 was the only one that showed a characteristic specific agglutination in either of the type sera.

In the original study which led to a serological classification of the disease-producing pneumococci, protection of animals against infection was adopted as probably the most specific of the immunological tests which could be applied, and it was only later, when agglutination was found to correspond very closely in specificity with the protection tests, that it was also accepted as a satisfactory method of differentiation. In table IV are presented the results obtained by testing the protective power of type sera against sputum pneumococci. White mice were given intraperitoneally varying doses of pneumococci, and at the same time a fixed quantity of immune serum. Animals surviving for five days were considered effectively protected. All animals except the virulence controls received 0.2 of a cubic centimeter of immune serum intraperitoneally. Serum of type I, in quantities of 0.2 of a cubic centimeter, uniformly protects against 0.1 of a cubic centimeter of a broth culture of the homologous organism, which kills mice regularly in doses of 0.000001 of a cubic centimeter. The same amount of serum of type II protects against 0.01 of a cubic centimeter of the homologous organism of similar virulence. The specimen of type II serum used in the following tests had a somewhat lower protective value than usual, but was sufficiently high to show dependable differences.

The results of the protective tests with thirteen of the fifteen strains of cocci studied are presented in table IV. The two organisms not tested were of such low virulence that it was impossible to kill mice with the moderate doses necessary for the successful carrying out of the test. In no instance has the protective value of the type sera been sufficiently high to justify placing any of the organisms studied in either of the fixed groups I or II. The irregular survivals occurring with the smallest doses of culture are somewhat difficult to explain. As is observed, these survivals sometimes occur among the control animals, with one or both of the specific immune

TABLE IV.

Protective Power of Type Sera for Pneumococci from Normal Sputum.

Dose of culture.	Strains of pneumococcus.									
	1			2			3 (*Pneumococcus mucosus*).			
	Controls.	Serum I.	Serum II.	Controls.	Serum I.	Serum II.	Controls.	Serum I.	Serum II.	Normal horse serum.
0.1 c.c.	.....	D. 14	D. 14	.....	D. 14	D. 14	.....	D. 20	D. 20	.....
0.01 c.c.	D. 18	D. 18	D. 18	D. 14	D. 18	D. 18	D. 20	D. 20	D. 20	S.
0.001 c.c.	.....	.....	.....	.....	.....	.....	D. 20	D. 20	D.20(?)	S.
0.0001 c.c.	D. 42	D. 18	D. 28	D. 28	S.	S.	S.	D. 48	D. 20	S.
0.00001 c.c.	D. 96	D. 28	S.	D. 36	S.	S.	S.	S.	D. 84	S.

	4			6				7			
	Controls.	Serum I.	Serum II.	Normal horse serum.	Controls.	Serum I.	Serum II.	Controls.	Serum I.	Serum II.	Normal horse serum.
0.1 c.c.	.....	D. 20	D. 20	.....	.....	D. 14	D. 18	.....	D. 20	D. 20	.....
0.01 c.c.	D. 20	D. 20	D. 20	D. 20	D. 18	D. 14	D. 18	D. 20	D. 20	D. 20	D. 20
0.001 c.c.	D. 20	D. 20	D. 20	D. 20	.....	.....	.....	D. 20	D. 20	D. 30	D. 20
0.0001 c.c.	D. 30	D. 20	D. 20	D. 20	D. 42	D. 24	D. 36	S.	D. 42	D. 20	D. 30
0.00001 c.c.	D. 42	D. 24	D. 30	D. 42	S.	D. 36	D. 42	S.	S.	D. 24	S.

	9				10			11		
	Controls.	Serum I.	Serum II.	Normal horse serum.	Controls.	Serum I.	Serum II.	Controls.	Serum I.	Serum II.
0.1 c.c.	.....	D. 20	D. 20	.....	.....	D. 18	D. 18	.....	D. 18	D. 18
0.01 c.c.	D. 20	D. 20	D. 30	D. 20	D. 18	D. 18	D. 18	D. 18	D. 18	D. 18
0.001 c.c.	D. 20	D. 20	D. 30	D. 20	.....	.....	.....	.....	.....	.....
0.0001 c.c.	S.	D. 36	D. 20	D. 30	D. 28	D. 18	D. 120	D. 72	D. 20	D. 20
0.00001 c.c.	S.	D. 42	S.	D. 30	D. 36	D. 36	S.	D. 66	D. 28	D. 120

	12			13			
	Controls.	Serum I.	Serum II.	Controls.	Serum I.	Serum II.	Normal horse serum.
0.1 c.c.	.....	D. 18	D. 18	.....	D. 20	D. 20	.....
0.01 c.c.	D. 18	D. 18	D. 18	D. 20	D. 20	D. 20	D. 20
0.001 c.c.	.....	.....	.....	D. 20	D. 20	D. 20	D. 20
0.0001 c.c.	D. 18	S.	D. 18	D. 20	D. 3(?)	D. 24	D. 25
0.00001 c.c.	D. 120	S.	S.	D. 72	D. 34	S.	D. 20

	14				15			
	Controls.	Serum I.	Serum II.	Normal horse serum.	Controls.	Serum I.	Serum II.	Normal horse serum.
0.1 c.c.	.....	S.	D. 20	.....	.....	D. 20	D. 20	.....
0.01 c.c.	S.	S.	S.	D. 20	D. 20	D. 20	D. 48	D. 20
0.001 c.c.	S.	S.	S.	S.	D. 20	D. 20	D. 20	D. 20
0.0001 c.c.	S.	S.	S.	S.	D. 30	D. 34	D. 20	D. 48
0.00001 c.c.	S.	S.	D. 72	S.	D. 34	D. 34	D. 24	D. 132

In the tables D. = died; S. = survived. The figures represent the number of hours before the death of the animal.

sera, and sometimes among the animals treated with normal horse serum. The average virulence of the sputum pneumococci for white mice is generally somewhat lower than that of pneumococci obtained from cases of lobar pneumonia. Our experience has been that among pneumococci of relatively low virulence variations in the effect of serum are often observed. In some instances the administration of serum seems to increase the infective power of the pneumococcus, and in other instances there may be a variable degree of protection that is apparently non-specific in character. Such effects are not manifest when highly virulent pneumococci from lobar pneumonia are used. For purposes of comparison a small number of such tests are shown in table V.

Table III shows that pneumococci 2, 9, 13, and 15 showed the nearest approach to specific agglutination, and we should expect some evidence of protective power with the corresponding sera, if the organisms in question belong definitely to the group in the sera of which agglutination occurred. Pneumococcus 2, which agglutinates slightly in serum I and strongly in serum II, shows a slight grade of protection with both sera; pneumococcus 9, which agglutinates slightly with serum I, shows no protection in either serum; pneumococcus 13, which develops strong agglutination only in serum II, shows a minimal degree of protection with the corresponding serum; pneumococcus 15 agglutinating slightly with serum I shows no protection with either of the type sera employed. These irregular crossings are not at present explainable, but they do not seem to indicate a close degree of specific relationship, such as is observed in the highly parasitic fixed types of pneumococcus. Raising the virulence when possible by animal passage does not change these organisms into typical representatives of any of the fixed types.

TABLE V.

Agglutination of Fifteen Strains of Pneumococcus from Lobar Pneumonia with Stock Sera of Groups I and II.

Culture of pneumococcus.	Group I.					Group II.					Group IV (heterogeneous).				
	A1	67	75	78	88	85	A3	A5	A10	A69	A38	A67	A71	A75	A78
Serum I...	++	++	++	++	++	....	....	....	....	....	....	....	....	....	....
Serum II...	....	....	....	....	....	++	++	++	+	++	....	....	....	....	....

Tables V and VI are presented to show how closely related are the strains of pneumococci obtained from lobar pneumonia to other members of the homologous group.

TABLE VI.

Protective Power of Type Sera for Pneumococci from Lobar Pneumonia.

Dose of culture.	Strains of pneumococci of group I.														
	A1			67			75			78			28		
	Controls.	Serum I.	Serum II.	Controls.	Serum I.	Serum II.	Controls.	Serum I.	Serum II.	Controls.	Serum I.	Serum II.	Controls.	Serum I.	Serum II.
0.1 c.c.	D. 72	S.	D. 72	D. 17	S.	D. 41	.....	D.120	.....	D. 18	D. 18	D. 18	.....	D. 18	.....
0.01 c.c.	D.144	S.	D. 96	D. 17	S.	D. 25	.....	S.	.....	D. 36	D. 20	D. 18	.....	S.	.....
0.001 c.c.	S.	S.	S.	D. 41	S.	D. 41	.....	S.	.....	D. 23	S.	D. 18	.....	S.	.....
0.0001 c.c.	D. 72	S.	D. 72	D. 41	S.	D. 41	D. 19	S.	D. 19	D. 19	S.	D. 20	D. 24	S.	D. 20
0.00001 c.c.	.....	.....	.....	D. 96	S.	D. 41	D. 30	S.	D. 24	D. 24	S.	D. 18	D. 20	S.	D. 22
0.000001 c.c.	.....	.....	.....	D. 48	S.	D. 72	D. 30	S.	D. 40	D. 36	S.	D. 36	D. 40	S.	D. 40

	Strains of pneumococci of group II.														
	85			A3			A5			A10			A69		
	Controls.	Serum I.	Serum II.	Controls.	Serum I.	Serum II.	Controls.	Serum I.	Serum II.	Controls.	Serum I.	Serum II.	Controls.	Serum I.	Serum II.
0.1 c.c.	.....	.....	D. 18	.....	.....	.....	.....	.....	.....	.....	D. 18	D. 30	.....	D. 18	D. 18
0.01 c.c.	.....	.....	S.	.....	.....	.....	.....	D. 18	S.	D. 19	D. 48	S.	.....	D. 18	S.
0.001 c.c.	D. 18	D. 18	S.	D. 18	D. 18	D. 24	.....	D. 18	S.	D. 30	D. 36	S.	.....	D. 18	S.
0.0001 c.c.	D. 18	D. 20	S.	D. 18	D. 18	S.	D. 30	D. 36	S.	D. 36	D. 48	S.	D. 18	.....	S.
0.00001 c.c.	D. 40	D. 22	S.	D. 18	D. 18	S.	D. 26	D. 18	S.	D. 36	D. 36	S.	D. 18	.....	S.
0.000001 c.c.	D. 40	S.	S.	D. 18	D. 18	S.	D. 26	.....	S.	.....	.....	.....	D. 18	.....	S.

	Strains of pneumococci of group IV (heterogeneous).														
	A38			A67			A71			A75			A78		
	Controls.	Serum I.	Serum II.	Controls.	Serum I.	Serum II.	Controls.	Serum I.	Serum II.	Controls.	Serum I.	Serum II.	Controls.	Serum I.	Serum II.
0.1 c.c.	.....	D. 18	.....	.....	D. 18	.....	.....	D. 18	.....	.....	D. 18	.....	.....	D. 18	D. 18
0.01 c.c.	.....	D. 24	.....	.....	D. 18	D. 24	.....	D. 18	D. 36	.....	D. 20	.....	.....	D. 20	D. 20
0.001 c.c.	D. 20	D. 22	D. 20	D. 22	D. 30	D. 18	D. 20	D. 18	D. 18	D. 18	D. 20	D. 18	D. 18	D. 24	D. 24
0.0001 c.c.	D. 48	D. 22	D. 48	D. 18	D. 36	D. 36	D. 25	D. 36	D. 25	D. 22	D. 21	D. 22	D. 22	D. 22	D. 22
0.00001 c.c.	D. 48	.....	D. 48	D. 36	.....	D. 36	D. 24	.....	D. 36	.....	.....	.....	.....	.....	.....
0.000001 c.c.	D. 48	.....	D. 48	D. 36	.....	.....	D. 36	.....	.....	.....	.....	.....	.....	.....	.....

Strains of pneumococcus isolated from patients with lobar pneumonia, as has been previously demonstrated, can be classified into

definite groups by means of their serological reactions. In tables V and VI are presented the agglutination and protection reactions of a small number of these strains belonging to groups I, II, and IV. The reactions of group III, the *Pneumococcus mucosus* group, are not given, because they are developed with some difficulty and have been discussed elsewhere. It is seen that groups I and II correspond exactly in their agglutination and protection reactions, and show practically no crossing. In group IV have been placed those organisms which do not react with serum I and II, and which by their growth characters are differentiated from *Pneumococcus mucosus*. Previous study has shown that individual members of this group show very little crossing in their serological reactions, and in this they resemble closely the pneumococci isolated from normal sputum. In addition, they are, as a rule, less pathogenic than members of the fixed groups.

Comparison of immunological reactions of pneumococci from normal sputum and of those from lobar pneumonia reveals certain facts which may have an important bearing on the epidemiology of this disease. In about 80 per cent. of cases of pneumonia, pneumococci are found which fall into well defined serological groups, and these organisms do not occur in normal mouths except in exceptional cases, limited, as far as our experience goes, to intimate contacts. Some evidence is therefore offered in support of the assumption that infection with one of the fixed types of pneumococcus represents contact infection, either direct or indirect, from some previous case of pneumonia, and not infection with a pneumococcus which habitually dwells upon the mucous membranes. On the other hand, about 20 per cent. of cases of pneumonia are due to heterogeneous pneumococci which cannot be differentiated from the strains occurring in normal sputum. It is possible that these infections are autogenic in character.

In order to demonstrate still further the differences between pneumococci occurring in disease and in the normal mouth, we have studied the disappearance of the fixed types from the sputum during convalescence from pneumonia, and the character of the pneumococcus which then becomes manifest. This study is being extended at the present time, and the results of only a few observations are

here presented. The pneumococci studied during recovery were obtained from the sputum and differentiated by the tests previously described. Observations made at varying intervals after the onset of the disease are presented in table VII.

TABLE VII.

Case.	Type of pneumococcus during height of disease.	Type of pneumococcus after recovery.
W.R.	Type I	60 dys. Type I.
		65 dys. Type IV.
A.W.	Type II	30 dys. Streptococcus.
		48 dys. Type IV.
		108 dys. Type IV.
A.	Type II	60 dys. Type IV.
S.H.	Type II	34 dys. Streptococcus.
		40 dys. Type IV.
K.W.	Type III	13 dys. Type III.
		73 dys. Type IV.
S.H.	Type II	47 dys. Streptococcus.
		78 dys. Type IV.
S.	Type I	59 dys. Streptococcus.
		73 dys. Streptococcus.
A.I.	Type II	53 dys. Type II.
McG.	Type I	30 dys. Type IV.
A.	Type I	90 dys. Type I.
U.	Type II	20 dys. Type IV.
		25 dys. Type IV.
B.	Type I	33 dys. Type IV.
S.	Type I	30 dys. Type IV.
M.S	Type II	30 dys. Type II.
C.	Type II	14 dys. Type IV.
B.	Type II	63 dys. Type II.
D.	Type II	21 dys. Type IV.
S.	Type II	24 dys. Type IV.
F.	Type I	12 dys. Type IV.
H.	Type II	15 dys. Streptococcus.

Study of the above table reveals the fact that only in exceptional instances does one find in the sputum, any considerable period of time after recovery, the type of pneumococcus with which the individual was infected during the disease. In four instances type organisms were still present at the time the patient was lost to observation, a period varying from thirty to ninety days after the onset of the disease. In two of these the signs of pneumonia persisted for a

long time and in one the type organism was obtained by lung puncture some three weeks after the onset of pneumonia. The pneumococcus can be isolated from the sputum of a large percentage of recovered cases, and this organism, except when the type strain has persisted, corresponds to the type of pneumococcus found in the mouths of normal individuals. The shortest period in which the type strain has been replaced by an organism of the sputum type has been twelve days after onset. Undoubtedly in a number of cases the first observations were made at too late a period to determine the exact time of disappearance of the type strain, and more carefully conducted studies are now showing that the type organisms disappear at an earlier period than table VII would lead one to suppose. The sputum type of pneumococcus obtained after recovery has been placed in what we term group IV, which consists, as has been stated previously, of a heterogeneous series of independent varieties according to our methods of classification. A systematic search for new fixed groups among these varieties is being carried on, but up to the present time no new group relationships have been discovered. From this study it is seen that the type organisms which are readily distinguishable during pneumonia are, in general, fairly rapidly supplanted by a pneumococcus which, as far as we can determine, corresponds to the type found in the sputum of normal individuals.

DISCUSSION.

As a result of the work described in this paper certain questions in regard to the etiology and epidemiology of lobar pneumonia present themselves for discussion. In order to produce disease, pathogenic microörganisms must gain entrance to the body through one of its exposed surfaces. Probably the commonest portal of entry is one of the mucous surfaces. It is well known that the mucous membranes of the body are constantly inhabited by a large variety of bacteria and that some of these microörganisms are closely related to certain strictly pathogenic types. Examples of such a condition are the Gram-negative, non-pathogenic diplococci, and the pathogenic and strictly parasitic gonococcus and meningococcus, also various bacilli of the gastro-intestinal group, and the typhoid bacillus. As a rule, the pathogenic members of these groups are readily dis-

tinguishable from their non-pathogenic relatives by a variety of simple reactions. Evidence is, however, gradually accumulating that the same condition of affairs exists among groups of organisms in which it is not so easy to differentiate the pathogenic from the non-pathogenic forms. It would seem probable that to this latter class belong the pneumococci, and that in this group we have strictly disease-producing types, and others which exist as more or less harmless saprophytes, which cannot be distinguished from the pathogenic members of the group, by the ordinary simple bacteriological methods. The studies reported in this paper show that about 80 per cent. of the pneumococci obtained from cases of lobar pneumonia belong to types which occur only in association with disease, and are not discoverable in the sputum of normal individuals. These seem to form the strictly parasitic types and are comparable to the true pathogens of certain other bacterial groups. In addition to these there are a smaller number of organisms associated with pneumonia which cannot be distinguished from the type of pneumococcus in normal sputum. Their lower virulence and general characters seem to indicate that they may be representatives of the type found in normal sputum. The more highly pathogenic forms are never found in normal sputum except in the case of intimate contacts. Wherever such contacts have been observed the type of pneumococcus in the contact has always corresponded exactly with that of the infected individual with whom he has been associated. These type organisms disappear from the mouths of contacts in the same manner as the same types disappear from the sputum of patients recovering from pneumonia.

It might be urged that the differences which we have demonstrated between various races of pneumococci are transient in character; that change from one type into another occurs with variation in environment. It is not possible at the present time to bring positive proof that such a phenomenon does not take place. Lyall[8] has recently isolated the pneumococci present in the deep sputum of a series of cases of pulmonary tuberculosis. In most instances these resembled the pneumococcus found in normal sputum. If change from the sputum type into the pathogenic types occurs with any

[8] Lyall, H. W., *Jour. Exper. Med.*, 1915, xxi, 146.

degree of readiness, one would expect to observe such changes under the conditions of this study, since in pulmonary tuberculosis the pneumococcus lives in the lung under conditions closely resembling those of lobar pneumonia. As our experience increases, we are more and more impressed with the constancy with which the varieties of pneumococcus retain their differential characters. No amount of animal passage, growth under a great variety of artificial conditions, or storage in a dried state has as yet caused the change of one type of pneumococcus into that of another, or the loss of the special characters by means of which it was originally classified. That the pneumococcus in its natural environment has reached a certain stage of equilibrium is fairly certain. In our experience we always obtain the same type of pneumococcus from normal sputum, and the same variety of groups from cases of lobar pneumonia. The pathogenic groups are now known to occur over widely separated areas of the globe. The status of bacterial mutation is so unsettled that in the absence of positive examples of permanent variations it has not seemed useful to us at the present time to make a study of the possibility of such changes among the pneumococci.

In view of the differences which seem to exist between pneumococci found in normal mouths and the dominant types occurring in lobar pneumonia, there is some interest in seeking an explanation of how they may be related to each other, and how the parasitic type has arisen from the more commonly saprophytic varieties. One might assume that today certain strains of pneumococcus are changing from more or less harmless organisms of the mouth to the disease-producing type found in pneumonia. The fact that a certain percentage of the organisms found in disease correspond more or less closely to those living in the normal mouth is some evidence in favor of this assumption. However, the majority of pneumococci which produce disease differ sharply from the type of organism found in normal sputum, and no new groups consisting of a large number of closely related strains have been found. If, then, the sputum type of pneumococcus rises to a high plane of parasitism, which enables it to produce a typical lobar pneumonia, it would seem to lose this quality after a single instance of infection, and not be able to perpetuate itself as a permanently pathogenic form and thus give rise to a number of instances of the disease.

We have recently received some strains of pneumococcus from South Africa, which seem to throw light on the manner in which new typical groups of pathogenic pneumococci arise, and the conditions that are necessary for these organisms to maintain their newly acquired disease-producing qualities. For a number of years pneumonia has been epidemic among the native mine laborers on the Rand. These men come for the most part from the more tropical regions of Africa where pneumonia is practically unknown, and they may be assumed to have a high degree of susceptibility to pneumococcus infection. The studies of Sir Almroth Wright[9] lend support to this assumption. As soon as the natives are brought to the Rand and come in contact with whites among whom pneumonia is fairly common, a high rate of incidence of pneumonia immediately develops among the blacks. Lister[10] has studied the strains of pneumococci here concerned and finds that they fall into five different groups showing specific immunological reactions. He has sent us five typical representations of these groups for comparison with the groups of pneumococci occurring in this country. Three of the strains give the characteristic reactions of members of the three typical groups found in New York. Two of the strains which belong to the dominant groups in South Africa have not as yet been met with here, and in view of the large number of organisms that have been examined by us, and from Neufeld's experience in Germany, it seems that they must be peculiar to South Africa. In other words, the condition in the Rand has been favorable to the origin of new fixed groups of pneumococcus. The most likely explanation of this phenomenon seems to be that in South Africa, among the whites, as in this country, there are a number of instances of pneumonia due to organisms resembling the sputum pneumococcus, the slightly virulent group IV of our classification. When such a pneumococcus is communicated to the susceptible black its pathogenic history, in spite of its relatively low virulence, does not stop with the production of a single instance of pneumonia, as it seems to among individuals whose racial immunity is relatively high, but the organism is readily passed on to other susceptible blacks, and

[9] Wright, A., *Lancet*, 1914, ii, 1, 87.
[10] Lister, F. S., *loc. cit.*

thus establishes itself in the less immune race as a permanently pathogenic type. If such is in reality the succession of events, then it is probable that permanent parasites arise only under conditions of high racial susceptibility, and that as the immunity of the race develops the ground becomes unfavorable for the occurrence of such a phenomenon, and no more new fixed races appear. Under the latter conditions the organisms of low pathogenicity may produce isolated instances of disease among especially susceptible individuals, but because of their low virulence and the increased racial immunity they can no longer establish themselves as permanently parasitic types. Where relative immunes are brought into contact with non-immunes, the condition, as far as the origin of new disease-producing races is concerned, is much the same as when the whole race is highly susceptible. It is possible that in South Africa the new races arising among the natives may after a number of generations gain sufficient virulence to become highly infectious for the relatively immune white.

SUMMARY.

A study of pneumococci isolated from individuals suffering from lobar pneumonia has shown that the majority of these organisms fall into definite biological groups. These groups have been arbitrarily numbered from I to IV. The first three groups consist of organisms which within the group are closely related to each other by certain immunological reactions; *i. e.,* protection and agglutination. Extensive study has failed to reveal crossing in either of these reactions between members of separate groups. The fourth group is formed of a series of independent varieties which cannot be definitely related to one another by the immune reactions employed. Up to the present time we have observed no tendency of these organisms to lose their specific characters, nor have we observed a change of one type into another. These groups vary in their pathogenicity for human beings, and in the order of their virulence are as follows: group III, group II, group I, group IV. The degree of protective power developed in the sera of animals immunized against members of the different groups varies inversely with the virulence and with the amount of capsular development. This, however, applies only

to tests of passive immunity. The highly virulent groups give as good active immunity as those of lower virulence, if not better.

In view of these constant differential characters of the pneumococcus, it was deemed advisable to study the pneumococci occurring in normal sputum. It has been commonly assumed that infection in pneumonia is autogenic, and occurs from the invasion of the lungs by a pneumococcus habitually carried in the mouth. If this is so, we should find the same types in the normal mouth as occur during the disease. Examination of a series of normal individuals showed this not to be the case. In no instance was an organism found which could be grouped with any of the fixed types of pneumococcus. All exhibited the same characters as those organisms obtained from lobar pneumonia which belong to group IV. Inasmuch as organisms belonging to this group are of low virulence, and are responsible in our experience for only 20 per cent. of the cases of pneumonia, it is at once manifest that the majority and more virulent cases of pneumonia are due to organisms which are not found in normal mouths. To gain further evidence of this difference, a study has been made of convalescents from pneumonia who had been infected by typical organisms. During the period of recovery these typical organisms are supplanted by the type which occurs in normal mouths. The period of disappearance of the typical varieties has varied. The shortest time in which disappearance has occurred has been twelve days, and the longest period in which typical organisms have been carried has been ninety days. In the latter instance the patient was lost sight of, so that he may well have carried the virulent form for a longer period of time. In general, when typical organisms persist for a long time, there is delay in the healing of the lung lesion. If recovery is prompt, as a rule the virulent types disappear rapidly.

We have said that the virulent types do not occur in normal mouths. There are exceptions to this observation. In a number of instances organisms belonging to the typical groups have been isolated from the mouth sputum of healthy individuals. So far this has occurred only in individuals intimately in contact with cases of lobar pneumonia. Wherever typical organisms have been obtained under such circumstances, the type has always corresponded to that with which the case of pneumonia was infected. Such individuals,

therefore, become infected with virulent types of pneumococcus by contact, and may be regarded as healthy carriers of disease-producing types.

This study makes it probable that the majority of cases of pneumonia are dependent upon either direct or indirect contact with a previous case. Mere infection of the mouth by virulent types is by no means sufficient to cause the disease. In order to invade the lungs, these virulent types must find the circumstances favorable, or a suitable condition must arise during the period when they are harbored in the mouth. Comparative study of certain strains of pneumococci received from South Africa suggests that new groups of parasitic organisms develop only during the period of high racial susceptibility. A like condition of affairs is brought about when a group of hitherto unexposed individuals is brought into contact with an infectious microörganism. The development of racial immunity soon limits the number of new types which may arise.

The suggestion is made that strictly parasitic races of microorganisms are pure lines and have established themselves as parasites during a period of high racial susceptibility.

THE DISTRIBUTION OF THE IMMUNE BODIES OCCURRING IN ANTIPNEUMOCOCCUS SERUM.*

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By a systematic study of the specific antigenic properties of the pneumococcus, Dochez and Gillespie[1] have demonstrated four distinct types of this organism occurring in disease. Such a biologic classification of pneumococci affords the only rational basis for the clinical application of serum therapy in lobar pneumonia. The immunologic and clinical studies of Cole[2] have shown that the protective and curative value of antipneumococcus serum is dependent upon this group specificity, that a serum to be efficacious must be one produced in response to an organism of the same type as that causing the infection. The treatment by specific antisera of lobar pneumonia of pneumococcus origin is at present possible only in infections due to organisms of types I and II. Immune serum produced by the third group, *Pneumococcus mucosus,* fails to confer passive immunity, and the heterogeneous nature of organisms of the fourth variety demands a specific serum for each individual strain, making serum therapy in infections of this type impracticable. However, since approximately 70 per cent. of the cases of this disease is due to organisms of the first two groups, the serum treatment of pneumonia is applicable in the majority of instances. Experimental evidence and clinical experience have demonstrated the necessity of administering relatively large doses of the appropriate serum in combating these infections. The present study was undertaken, therefore, with the hope of determining a method of concentrating and purifying antipneumococcus serum, by which its antibacterial potency might be conserved with a minimum of foreign protein. It seems reasonable to assume that such a process, by concentration of antibody content, might enhance the efficacy of

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[1] Dochez, A. R., and Gillespie, L. J., *Jour. Am. Med. Assn.,* 1913, lxi, 727.

[2] Cole, R., *Arch. Int. Med.,* 1914, xiv, 56.

serum treatment, and, by the elimination of certain irrelevant protein constituents, lessen the incidence, or at least ameliorate the symptoms of serum disease.

Although much is known concerning the immunological reactions of various immune bodies, comparatively little is understood of their true chemical nature. Whether they are themselves protein in character, or merely associated in some obscure chemical combination with the globulin or albumin of immune sera is not definitely known. The fact that they may be thrown out of solution by certain protein precipitants has been utilized in the concentration of antitoxic sera. Most investigators now agree that antibody precipitation by chemical agents is not merely a mechanical or adsorption phenomenon, but that these immune substances partake of the nature of the protein with which they are precipitated.

In the present study it was first necessary, therefore, to determine with which fraction of the serum protein the pneumococcus immune bodies are associated. The serum used in these experiments was obtained by the intensive immunization of horses to virulent cultures of pneumococcus of types I and II. The various protein fractions isolated were tested for antibacterial action against virulent cultures of pneumococcus by protection experiments on white mice, and the results compared with the similar power of the whole serum. This method is less accurate than the more exact titration of antitoxic sera. The potency of antipneumococcus serum can be measured only by its ability to protect highly susceptible animals against actual infection, and the comparative antibody content of any given serum fraction can be interpreted only in terms of the death or survival of such animals. This reaction between antibody and bacterium is biological and not chemical, as in the neutral balance of antitoxin and toxin mixtures. Under these experimental conditions, however, some information has been gained concerning the distribution of the immune bodies occurring in antipneumococcus serum.

Experiment 1.—To determine the protective value of the total globulins precipitated by half saturation with ammonium sulphate.

300 c.c. of antipneumococcus serum I were diluted with an equal volume of water, and 600 c.c. of a saturated solution of ammonium sulphate were added.

The mixture was allowed to stand at room temperature over night, filtered, the precipitate collected, pressed, and dialyzed for eight days against running tap water. To the filtrate crystals of ammonium sulphate were added to full saturation. The precipitate consisting of albumin was filtered off, pressed, and dialyzed as above.

The volume of globulin solution was 100 c.c., so that the globulins were three times as concentrated as in the whole serum. The volume of albumin solution was 58 c.c., being 0.2 per cent. that of the original serum.

Protective Power of Globulins, Albumin, and Serum I.
Pneumococcus I.

Amount of culture I.	Amount of serum fractions.	Serum I.	Globulins.	Albumin.	Controls, Culture alone.
0.05 c.c.	0.1 c.c.	S.	S.	D. 18	.....
0.05 c.c.	0.08 c.c.	S.	S.	D. 18	.....
0.05 c.c.	0.05 c.c.	S.	S.	D. 18	.....
0.05 c.c.	0.03 c.c.	D. 72	S.	D. 18	.....
0.05 c.c.	0.01 c.c.	D. 48	D. 72	D. 18	.....
0.0001 c.c.	.....	.....	.....	.....	D. 36
0.00001 c.c.	.....	.....	.....	.....	D. 36
0.000001 c.c.	.....	.....	.....	.....	D. 36

In the tables D. = died; S. = survived. The figures represent the number of hours before the death of the animal.

Experiment 2.—Antipneumococcus serum II was used, diluted with an equal volume of water, and the globulins were precipitated by half saturation with ammonium sulphate. The albumin was precipitated from the filtrate by complete saturation with crystals of ammonium sulphate. Both fractions were dialyzed for four days against running tap water, and made up to the original volume of serum with 0.85 per cent. salt solution.

Comparative Protective Value of Globulins, Albumin, and Serum II.
Pneumococcus II.

Amount of culture II.	Amount of serum fractions.	Serum II.	Globulins.	Albumin.	Controls. Culture alone.
0.1 c.c.	0.2 c.c.	D. 66	D. 66	D. 18	.....
0.01 c.c.	0.2 c.c.	S.	S.	D. 19	.....
0.001 c.c.	0.2 c.c.	S.	S.	D. 19	.....
0.0001 c.c.	0.2 c.c.	S.	S.	D. 24	D. 19
0.00001 c.c.	0.2 c.c.	S.	S.	D. 24	D. 24
0.000001 c.c.	0.2 c.c.	S.	S.	D. 36	D. 24

Agglutination.

Dilution.	Serum II.	Globulins.	Albumin.	Controls.
1 : 1	++	++	−	−
1 : 10	++	++	−	−
1 : 20	+	±	−	−
1 : 40	−	−	−	−

Experiment 3.—The agglutination test was made by mixing equal volumes of an eighteen hour broth culture of pneumococcus I and varying dilutions of serum fractions.

The antigen used in the precipitin reaction was prepared from the washed bacterial residue of liter cultures of pneumococcus I frozen and desiccated in vacuum over sulphuric acid. The dry powder was dissolved in salt solution, with 1 mg. per c.c., and a 1 : 10 dilution of this was used in the test. Equal parts of antigen and serum dilutions were mixed and incubated in the water bath at 37° C. for two hours.

Agglutination and Precipitation.

Agglutination.				Precipitation.		
Serum dilution.	Serum I.	Globulins.	Albumin.	Serum dilution.	Serum I.	Globulins.
1 : 1	++	++	−	1 : 10	++	++
1 : 10	++	++	−	1 : 15	++	++
1 : 100	−	−	−	1 : 20	++	+
				1 : 25	+	±
				1 : 30	±	−

From experiments I, II, and III it may be concluded that the immune bodies of antipneumococcus serum are not dialyzable, that they are precipitated with the globulins by half saturation with ammonium sulphate, and that the demonstrable antibodies, such as agglutinins, precipitins, and protective substances, are combined or associated with the globulins and not with the albumin of the immune serum.

Experiment 4.—To determine the protective value of the euglobulin and pseudoglobulin.

300 c.c. of antipneumococcus serum I were diluted with an equal volume of water and 600 c.c. of a saturated solution of ammonium sulphate added, allowed to stand over night, and filtered, precipitate (a), filtrate (a). The precipitate (a) containing both globulins was taken up in 600 c.c. of water and saturated with crystals of sodium chloride (210 gm.) and filtered, precipitate (b), filtrate (b). The precipitate (b) was again taken up in water and resaturated with salt, the final precipitate pressed and dialyzed against running tap water until salt-free. The dialysate was made slightly alkaline to effect solution, used undiluted, and called "euglobulin." To the filtrate (b) about 0.2 per cent. of acetic acid was added to precipitate the globulin remaining in solution. This precipitate was pressed and after dialyzing for twenty-four hours was neutralized to litmus by sodium carbonate, and dialysis continued for four days. The dialysate was used undiluted and called "pseudoglobulin." The original filtrate (a) was saturated with crystals of ammonium sulphate; the precipitate containing the albumin was collected by filtration, pressed, and dialyzed.

Comparative Protective Value.

Amount of culture I.	Amount of serum fractions.	Serum I.	Euglobulin.	Pseudoglobulin.	Albumin.	Controls.
0.1 c.c.	0.2 c.c.	D. 72	D. 17	D. 20	D. 17	.....
0.01 c.c.	0.2 c.c.	S.	S.	D. 84	D. 17	.....
0.001 c.c.	0.2 c.c.	S.	S.	S.	D. 20	.....
0.0001 c.c.	0.2 c.c.	S.	S.	S.	D. 24	D. 22
0.00001 c.c.	.....	.....	.....	.....	.....	D. 24
0.000001 c.c.	.....	.....	.....	.....	.....	D. 36

Amount of culture I.	Amount of serum fractions.	Serum I.	Euglobulin.	Pseudoglobulin.	Albumin.	Controls.
0.01 c.c.	0.4 c.c.	S.	S.	S.	D. 48	.....
0.01 c.c.	0.3 c.c.	S.	S.	S.	D. 36	.....
0.01 c.c.	0.2 c.c.	S.	S.	S.	D. 18	.....
0.01 c.c.	0.1 c.c.	S.	S.	S.	D. 28	.....
0.01 c.c.	0.01 c.c.	D. 40	D. 40	D. 40	D. 18	.....
0.01 c.c.	0.005 c.c.	D.	D. 18	D. 48	D. 18	.....
0.0001 c.c.	.....	.....	.....	.....	.....	D. 18
0.00001 c.c.	.....	.....	.....	.....	.....	D. 28
0.000001 c.c.	.....	.....	.....	.....	.....	D. 36

Experiment 4 shows that by the technique employed the immune bodies of antipneumococcus serum are not confined to the so called euglobulin or pseudoglobulin, but occur in both these fractions. Their absence in the albumin of the serum confirms the previous experiment.

Experiment 5.—Fractional precipitation of heated serum by ammonium sulphate.

50 c.c. of antipneumococcus serum I were diluted with a half volume of distilled water and 1 per cent. sodium chloride, and 38.6 c.c. of a saturated solution of ammonium sulphate were added, making the total 34 per cent. saturation. The serum-sulphate mixture was heated in a water bath at 56° C. for four hours, 58° C. for one hour, and 60° C. for five minutes. Filtered hot, the precipitate was pressed and dialyzed for four days against running water. To the filtrate, a saturated solution of ammonium sulphate was added up to 54 per cent. saturation. After standing at room temperature over night the precipitate was filtered off, pressed, and dialyzed for four days. To the final filtrate ammonium sulphate crystals were added to complete saturation, and the filtrate was pressed and dialyzed. The various fractions isolated were designated 34 per cent. globulin, 54 per cent. globulin, and albumin, respectively. After dialysis these fractions were each made up with 0.85 per cent. salt solution to the volume of the original serum, and in the case of the 34 per cent. globulin sufficient sodium carbonate was added to effect solution.

Comparative Protective Value of Globulin Fractions Precipitated by Ammonium Sulphate from Heated Serum.

Amount of culture I.	Amount of serum fractions.	0–34% globulin.	34–54% globulin.	Albumin.	Whole globulins, experiment 1.	Serum I.	Controls. Culture alone.
0.1 c.c.	0.2 c.c.	D. 20	D. 20	D. 20	S.	S.	.....
0.01 c.c.	0.2 c.c.	S.	S.	D. 20	S.	S.	.....
0.001 c.c.	0.2 c.c.	S.	S.	D. 20	S.	S.	.....
0.0001 c.c.	0.2 c.c.	S.	S.	D. 20	S.	S.	D. 18
0.00001 c.c.	0.2 c.c.	S.	S.	D. 24	S.	S.	D. 18
0.000001 c.c.	0.2 c.c.	S.	S.	D. 24	S.	S.	D. 18

Agglutination tests showed the presence of these antibodies in both fractions, and their absence in the albumin.

The method followed in this experiment is essentially that devised by Banzhaf[3] for the concentration of diphtheria antitoxin. In diphtheria immune serum the antitoxin is precipitated with the pseudoglobulin. The addition of salt and the heating of serum-sulphate mixture converts some of the pseudoglobulin into the inactive euglobulin without material loss of its antitoxic potency. By the application of this method to antipneumococcus serum it was hoped that the residue of protective antibodies occurring in the pseudoglobulin might be changed over with the converted euglobulin and the total potency of the serum be thus concentrated in the latter fraction. This, however, did not occur; both fractions still showed protection. Heating antipneumococcus serum to the temperature used in this experiment does not seem materially to affect the activity of the agglutinins and protective antibodies, although some loss of potency is suffered by the latter.

Experiment 6.—To determine the protective value of the globulin fraction precipitated by passage of carbon dioxide through diluted serum.

50 c.c. of antipneumococcus serum II were diluted with ten volumes of distilled water. Carbon dioxide was allowed to bubble through slowly for two hours, the diluted serum being kept at a temperature of 2° C. during the process; it was then placed in the ice box over night, and a sharp separation of precipitate and supernatant fluid was effected by centrifugalization. The carbon dioxide precipitate was dissolved in 50 c.c. of 0.85 per cent. salt solution. The supernatant fluid was half saturated with ammonium sulphate by the addition of an equal volume of a saturated solution of this salt. This carried down the residual globulin remaining after removal of the carbon dioxide-insoluble

[3] Banzhaf, E. J., *Studies from the Research Laboratory, Department of Health*, New York, 1912–13, vii, 114.

fraction. After standing at room temperature over night the precipitate was filtered, pressed, and dialyzed for four days and made up to a volume of 50 c.c. with 0.85 per cent. salt solution.

Protective Value of Carbon Dioxide Globulins.

Amount of culture II.	Amount of serum fractions.	Original serum II.	Carbon dioxide-insoluble globulin.	Carbon dioxide-soluble globulin.	Controls. Culture alone.
0.1 c.c.	0.2 c.c.	D. 36	D. 36	D. 18	.....
0.01 c.c.	0.2 c.c.	S.	S.	S.	.....
0.001 c.c.	0.2 c.c.	S.	S.	S.	.....
0.0001 c.c.	0.2 c.c.	S.	S.	S.	D. 20
0.00001 c.c.	0.2 c.c.	S.	S.	S.	D. 18
0.000001 c.c.	0.2 c.c.	S.	S.	S.	D. 18

Agglutination.

Dilution of serum fractions.	Original serum II.	Carbon dioxide-insoluble globulin.	Carbon dioxide-soluble globulin.
1 : 1	++	++	++
1 : 5	++	++	+
1 : 10	+	+	−
1 : 15	±	−	−
1 : 25	−	−	−

Precipitation.

Antigen 1:1,000.

Dilution of serum fractions.	Original serum II.	Carbon dioxide-insoluble globulin.	Carbon dioxide-soluble globulin.
1 : 1	++	++	+±
1 : 5	+	+	−
1 : 10	±	± (?)	−

From experiment 6 it appears that from diluted antipneumococcus serum there is precipitated by saturation with carbon dioxide a globulin possessing high protective value, and that by the method of titration employed a slightly less potent globulin fraction remains in solution, and can be separated from the albumin by ammonium sulphate. The somewhat anomalous fact, that each fraction of the carbon dioxide globulin apparently possesses protective value equal to the whole serum, may be explained in part by the method of titration. The immunity unit, 0.2 of a cubic centimeter of serum, which has been found most suitable for protection tests, apparently contains an excess of antibody and the zone of carbon

dioxide precipitation may so lie within the limits of this excess that, in the cleavage effected by acidification with carbon dioxide, sufficient antibody is carried over with each fraction of the globulin to afford an equivalent protection. The carbon dioxide-insoluble globulin, although representing approximately only one-fifteenth of the total protein, apparently contains more than half the antibodies.

Experiment 7.—To determine the potency of the serum globulins soluble and insoluble in saturated sodium chloride.

50 c.c. of antipneumococcus serum II diluted with an equal volume of water were saturated with crystals of sodium chloride, and the salt-serum mixture was allowed to extract over night at room temperature. The portion of the globulin insoluble in saturated sodium chloride was filtered off, pressed, and dialyzed for four days against running water. To the filtrate containing the salt-soluble fraction was added an equal volume of a saturated solution of ammonium sulphate. The precipitate containing the salt-soluble globulin was collected, pressed, and dialyzed for four days. The fractions were all diluted with 0.85 per cent. salt solution to the volume of the original serum.

Protective Value of Sodium Chloride Globulins.

Amount of culture II.	Amount of serum fractions.	Original serum II.	Sodium chloride-insoluble globulin.	Sodium chloride-soluble globulin.	Controls. Culture alone.
0.01 c.c.	0.2 c.c.	S.	D. 20	S.	.....
0.001 c.c.	0.2 c.c.	S.	S.	S.	.....
0.0001 c.c.	0.2 c.c.	S.	S.	S.	D. 20
0.00001 c.c.	0.2 c.c.	S.	S.	S.	D. 24
0.000001 c.c.	0.2 c.c.	S.	S.	S.	D. 36

Agglutination.

Serum II	++
Sodium chloride-insoluble	++
Sodium chloride-soluble	++

Experiment 7 shows that the protective substances and the agglutinins of antipneumococcus serum are not confined to either of the globulin fractions separated by saturation with sodium chloride. The salt-insoluble globulin, according to Freund and Joachim,[4] represents the euglobulin, but includes only a part of the similar fraction precipitated by one-third saturation with ammonium sulphate. Banzhaf and Gibson[5] consider that this method yields as

[4] Freund, E., and Joachim, J., *Ztschr. f. physiol. Chem.*, 1902, xxxvi, 407.

[5] Banzhaf, E. J., and Gibson, R. B., *Studies from the Research Laboratory, Department of Health*, New York, 1908–10, iv–v, 202.

sharp a differentiation as possible between the two globulin fractions, and that the sodium chloride separation probably represents more truly the common conception of euglobulin and pseudoglobulin than the more usual fractioning by ammonium sulphate. The results obtained in this experiment are similar to and comparable with those of experiment 4, in which the globulins were first precipitated with ammonium sulphate, and then separated by precipitation first with sodium chloride and then with acetic acid.

Experiment 8.—To determine the potency of the water-soluble and -insoluble serum globulin obtained by dialysis.

100 c.c. of antipneumococcus serum II, undiluted, were placed in a parchment bag and dialyzed for six days against running tap water. The dialyzing bag was then washed out with distilled water, the precipitate collected by filtration, washed with water, taken up in 100 c.c. of physiological salt solution, and made slightly alkaline with sodium carbonate to effect complete solution. To the filtrate containing the water-soluble globulin, an equal volume of a saturated solution of ammonium sulphate was added, the filtrate collected, pressed, and dialyzed for four days, and then made up to volume (100 c.c.) with 0.85 per cent. salt solution.

Protective Value of Globulin Fractions Separated by Dialysis.

Amount of culture II.	Amount of serum fractions.	Water-soluble globulin.	Water-insoluble globulin.	Original serum II.	Controls.
0.01 c.c.	0.2 c.c.	D. 24	S.	S.	.....
0.001 c.c.	0.2 c.c.	S.	S.	S.	.....
0.0001 c.c.	0.2 c.c.	S.	S.	S.	D. 20
0.00001 c.c.	0.2 c.c.	S.	S.	S.	D. 24
0.000001 c.c.	0.2 c.c.	S.	S.	S.	D. 40

Agglutination.

Pneumococcus II.	Serum II.	Water-insoluble globulin.	Water-soluble globulin.
	++	++	++

Seng[6] showed that by dialysis of diphtheria antitoxin only a small part (1/23 to 1/11) of the total globulin was insoluble and that this fraction possessed no antitoxic value. These results were later confirmed by Brieger and Krause.[7] Freund and Joachim do not consider the globulins precipitated by fractionation with ammonium sulphate identical with the two groups obtained by dialysis.

[6] Seng, W., *Ztschr. f. Hyg. u. Infektionskrankh.*, 1899, xxxi, 513.

[7] Brieger, L., and Krause, M., *Berl. klin. Wchnschr.*, 1907, xliv, 946.

Experiment 8 demonstrates the distribution of the antibacterial substances of antipneumococcus serum in both fractions of the globulins separated by dialysis. These results indicate that the globulin precipitated by dialysis (water-insoluble), like that precipitated by carbon dioxide, contains a part, but not all of the immune bodies.

Experiment 9.—To determine the relative potency of the globulin fractions obtained by the addition of progressive amounts of ammonium sulphate.

50 c.c. of antipneumococcus serum II were diluted with a half volume of water and the whole was precipitated by 34 per cent. saturation with ammonium sulphate, 38.6 c.c. of saturated solution. From the filtrate the residual globulin was precipitated by adding sufficient saturated solution of ammonium sulphate to cause 54 per cent. saturation, allowing for the amount of this salt already present in the filtrate. The precipitates of both fractions were collected, pressed, and dialyzed for four days against running tap water. The dialysates were made up to the original volume of the serum with 0.85 per cent. salt solution, and were designated 0 to 34 per cent. globulin and 34 to 54 per cent. globulin, respectively. By the same technique a similar quantity of the same lot of serum was fractioned by salting out at 38 and 42 per cent. saturation, the residual globulin in each case being separated from the albumin in the filtrate by precipitation at 54 per cent. saturation ammonium sulphate.

Relative Potency of the Globulins Obtained by Salting Out with Progressive Amounts of Ammonium Sulphate.

Amount of culture II.	Amount of serum fractions.	Controls. Culture alone.	Globulins precipitated by ammonium sulphate saturation within the limits						
			0–34%	34–54%	0–38%	38–54%	0–42%	42–54%	Original serum.
0.01 c.c.	0.2 c.c.	.....	S.	D. 20	S.	D. 22	S.	D. 18	S.
0.001 c.c.	0.2 c.c.	.....	S.	D. 72	S.	D. 24	S.	D. 24	S.
0.0001 c.c.	0.2 c.c.	D. 36	S.	D. 18	S.	D. 36	S.	D. 36	S.
0.00001 c.c.	0.2 c.c.	D. 36	S.	S.	S.	D. 36	S.	D. 36	S.
0.000001 c.c.	0.2 c.c.	D. 36	S.	S.	S.	D. 36	S.	D. 36	S.

Agglutination with Various Globulin Fractions.

Serum II, lot 3	++
0–34 per cent. globulin	++
34–54 per cent. globulin	±
0–38 per cent. globulin	++
38–54 per cent. globulin	—
0–42 per cent. globulin	++
42–54 per cent. globulin	—

Experiments 1 to 8 show that the immune bodies occurring in antipneumococcus sera I and II are associated or combined with the globulins, but are not confined solely to any one of the globulin

fractions obtained by the various methods of separation employed. Since certain of the experiments seemed to indicate that the major portion of the antibacterial substances are crowded toward the true or euglobulin end of the protein spectrum, an attempt was made in experiment 9 to determine, by fractional salting out with progressive amounts of ammonium sulphate, a point where the zones of globulin and antibody precipitation might coincide and the total potency of the serum be conserved with a minimum of serum globulin. Experiment 9 indicates that this zone of joint precipitation of total antibody with minimal globulin is reached at about 38 per cent. saturation with ammonium sulphate. At this point a fraction of the serum globulin is precipitated which is as active in antibacterial potency as the original serum, while the residual globulin is inactive and apparently unessential.

DISCUSSION.

The chemistry of the proteins of immune serum and their relation to various antibodies have been studied thoroughly in the antitoxic sera. Numerous investigators have shown that diphtheria and tetanus antitoxin are precipitated with the serum globulins. More exact study of these globulins has demonstrated that they may be further subdivided according to their solubility and precipitation by certain chemical reagents. Seng showed that the globulins of immune serum, and according to Marcus[8] those of normal serum as well, are of two kinds: an insoluble globulin precipitated by acetic acid, carbon dioxide, dilution with water, or dialysis; and a soluble globulin unaffected by these reagents but precipitated by the neutral salts of ammonium and magnesium sulphate. Seng showed, and many investigators have since confirmed, the association of antitoxin with the soluble globulin. Fractional precipitation with the neutral salts of the heavy metals yields a similar, though not altogether comparable, separation of the globulins into a soluble and insoluble fraction, the so called euglobulin and pseudoglobulin. It is with the latter of these, the pseudoglobulin, that antitoxin is combined. By the application of this principle Gibson and Banzhaf have devised an efficient and economic method for the concentration of diphtheria antitoxin.

[8] Marcus, E., *Ztschr. f. physiol. Chem.*, 1899, xxviii, 559.

It is now generally accepted that the antibodies occurring in immune serum are protein in character and are not readily dissociated from the protein of the serum in which they occur. Pröscher's[9] attempt to produce a non-protein antitoxin by digestion of antidiphtheria serum with trypsin has not been confirmed. Banzhaf[10] and Mellanby found that the ratio of protein digestion and antibody destruction are approximately the same. Seng, Joachim,[11] Atkinson,[12] Ledingham,[13] and Banzhaf found that certain quantitative changes occur in the serum proteins of animals immunized to bacterial toxins, and that the increase in the globulin content bears a marked relation to the increase in antitoxic potency of the serum. Landsteiner[14] has shown that the antitryptic action of normal serum is associated with the albumin fraction, and Opie and Barker[15] have found antileucoprotease in the same fraction.

The distribution of immune bodies in the blood serum of different animal species varies. Pick[16] found that in immune horse serum diphtheria antitoxin was associated with the pseudoglobulin, while in goat serum it is present in the euglobulin. The lysins and agglutinins of cholera occur in the euglobulin fraction of the serum of immunized goats and horses. Typhoid agglutinins in the immune serum of the horse are found in the pseudoglobulin, while in goats, rabbits, and guinea pigs they occur only in the euglobulin. Hartley[17] has recently shown that the immune bodies of antirinderpest serum are present in the euglobulin fraction of bovine serum.

The present work on the distribution of the immune bodies of antipneumococcus serum is confined to a study of their occurrence in the blood serum of horses immunized to types I and II of the pneumococcus. The sera were produced by repeated intravenous injections of these organisms, beginning with dead bacteria and

[9] Pröscher, *München. med. Wchnschr.*, 1902, xlix, 1176.
[10] Banzhaf, E. J., *Bull. Johns Hopkins Hosp.*, 1911, xxii, 106.
[11] Joachim, J., *Arch. f. d. ges. Physiol.*, 1903, xciii, 558.
[12] Atkinson, J. P., *Jour. Exper. Med.*, 1900–01, v, 67.
[13] Ledingham, J. C. G., *Jour. Hyg.*, 1907, vii, 65.
[14] Landsteiner, K., *Centralbl. f. Bakteriol., Ite Abt., Orig.*, 1900, xxvii, 357.
[15] Opie, E. L., and Barker, B. I., *Jour. Exper. Med.*, 1907, ix, 207.
[16] Pick, E. P., *Beitr. z. chem. Phys. u. Path.*, 1902, i, 351.
[17] Hartley, P., *Memoirs of the Department of Agriculture*, India, 1913–14, i, 177.

progressively increasing the doses, until the animals were able to tolerate the bacterial residue of a liter of live virulent pneumococcus injected directly into the blood stream. The serum was obtained from horses which had been immunized over periods of one to two years. The method employed in obtaining antipneumococcus serum differs entirely from that used in the production of antitoxins. In the former, the immunizing response is provoked by a live virulent organism, while in the latter the stimulus is in the nature of a bacteria-free toxin. The immunity mechanism involved in the elaboration of specific antipneumococcus substances may or may not differ physiologically from that concerned in the production of antitoxins, but the types of antibody evoked in each instance are distinct, and their distribution in the serum globulins is different.

SUMMARY.

The immune bodies of antipneumococcus serum are completely precipitated by 38 to 42 per cent. saturation with ammonium sulphate.

They are incompletely precipitated by (a) ammonium sulphate in less than 38 per cent. saturation, (b) saturation with sodium chloride, (c) dilution and saturation with carbon dioxide, (d) removal of crystalloids by dialysis.

The immune bodies of antipneumococcus serum are, therefore, associated or combined with that fraction of the globulins precipitated by 38 to 42 per cent. saturation with ammonium sulphate. The immune body fraction does not correspond exactly with the ordinary euglobulin (one-third saturation with ammonium sulphate or complete saturation with sodium chloride) or with the insoluble globulins precipitated by carbon dioxide or dialysis. These fractions carry with them only a part of the immune bodies.

Neither the albumin nor that fraction of the globulin not precipitated by 38 to 42 per cent. saturation of ammonium sulphate contain any of the demonstrable antibodies.

The most promising method for the practical purification of the immune bodies occurring in antipneumococcus serum appears to be precipitation by 38 to 42 per cent. saturation with ammonium sulphate.

THE TYPES OF PNEUMOCOCCI IN TUBERCULOUS SPUTUM.*

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The success of Cole[1] and his associates[2] in establishing four groups among the pneumococci affords a new basis for the study of the transmissibility and pathology of acute lobar pneumonia. The most important result of this work is, perhaps, its bearing on the epidemiology of the disease. It has been generally accepted that the pneumococci of the normal mouth may, under favorable conditions, become implanted in the lung and are there able to set up the condition known as lobar pneumonia. The work of Rosenow[3] has been considered as favoring this theory by supposing that the pneumococci of the mouth, under changed environmental conditions, undergo a mutation into a virulent type. On the other hand, the work indicating that different types of pneumococci may operate in producing pneumonia, raises the question as to whether pneumonia may not be a transmissible or epidemic disease caused by distinct, virulent types of this organism, passed from individual to individual. To prove the first theory, it must be shown either that the organism of acute pneumonia and that ordinarily present in the healthy mouth are the same, or, postulating a mutation, that the comparatively avirulent mouth strain, as a result of the changed lung condition, assumes the characteristics of one of the virulent groups associated with the acute disease.

It was with this problem in mind that the present work was undertaken. In pulmonary tuberculosis and the other lung conditions

* Received for publication, November 27, 1914.

[1] Cole, R., Pneumococcus Infection and Lobar Pneumonia, *Arch. Int. Med.*, 1914, xiv, 56.

[2] Dochez, A. R., and Gillespie, L. J., A Biologic Classification of Pneumococci by Means of Immunity Reactions, *Jour. Am. Med. Assn.*, 1913, lxi, 727.

[3] Rosenow, E. C., Transmutations within the Streptococcus-Pneumococcus Group, *Jour. Infect. Dis.*, 1914, xiv, 1.

represented in the present series of cases there is to be found, perhaps, the nearest approach to the pathological condition found in the pneumonic lung. If the changed condition of the lung tissue is to be regarded as the important factor in the transformation of the pneumococci ordinarily present in the mouth into the virulent organisms of acute pneumonia, it might be reasonably expected that the similarly changed conditions in tuberculosis would bring about the same result. If, in a study of such cases, it were to be found that the virulent groups I and II predominated, this would be strong evidence that the access to pathological lung tissue called forth a definite change in the biological characteristics of the pneumococcus.

In the present series fifty cases were studied with a view to determining the type of pneumococcus present in the sputum. These cases were, with one exception, patients at the New York State Hospital for Incipient Tuberculosis. The one exception, case 32, upon examination proved to be acute bronchial asthma. Of the remaining forty-nine, forty-three were cases of frank pulmonary tuberculosis, five were diagnosed as bronchiectasis (probably non-tuberculous), and one was a case of chronic asthma in which the diagnosis of tuberculosis had not been definitely made. The fact that all patients had received sanatorium treatment for varying lengths of time might conceivably have had some influence on the bacterial flora of the mouth or sputum.

In the selection of patients the more advanced cases of tuberculosis were purposely chosen. As judged by the Turban scale, the Gaffky count, and the general physical findings in the monthly examinations, the forty-three cases were distributed as follows: stage 1, three; stage 2, nineteen; and stage 3, twenty-one. Of these, eighteen were cavity cases, and eleven were subject to recurrent hemoptysis. Whenever possible, these latter cases were examined during the height of the hemorrhage. The five cases of bronchiectasis were of long duration, with copious expectoration, and all were probably non-tuberculous. One of the two cases of asthma was of the chronic type, complicated with some symptoms which were suspicious of tuberculosis. The other was acute bronchial asthma. Only three gave a history of a previous pneumonia, two of which were recent. It is interesting to note that from case 43, with a

history of pneumonia in January, 1914, an organism of group I was isolated.

The following routine procedure was adopted. The first sample examined was that of the unwashed sputum. This consisted of deep morning sputum, expectorated directly into a sterile Petri dish. This was carried to the laboratory and carried through as quickly as possible, as follows:

A small piece of sputum, about the size of a pea, was rubbed up in a sterile mortar with a little nutrient bouillon. 0.5 to 1 c.c. of this mixture was injected into the abdominal cavity of a white mouse weighing 15 to 20 gm. Upon the death of the animal, which usually occurred inside of forty-eight hours, an aseptic autopsy was performed and cultures were made from the peritoneal exudate and heart's blood on plain blood agar and plain beef broth (reaction +0.3 to +0.6 per cent. to phenolphthalein). In practically all cases, a pure culture of the infecting organism was obtained. In addition to the cultures, the spleens of all animals were saved and preserved by desiccation. In this way a stock culture was retained in case of an accident to the broth or agar culture. Capsule and Gram stains were done on the peritoneal exudates and heart's blood of all animals. The washed specimen was treated in the same manner, except that it was first thoroughly washed through several changes of sterile saline, according to the Kitasato method as modified by the Saranac Lake Laboratory.

All organisms, when finally isolated in pure culture, were tested for their power to ferment inulin, to coagulate litmus milk, and for their solubility in bile. If these three tests were positive, and the organism showed a capsule, agglutination tests with sera I and II were done, according to the technique used at The Rockefeller Institute Hospital. Finally, pathogenicity and protection tests were carried out with each organism. However, before this was done, the virulence of the strains was increased by mouse passage. This is necessary with the majority of sputum strains in order to insure reasonably uniform results in the protection experiments. The protection was carried out in accordance with the routine technique used at The Rockefeller Institute Hospital, and was as follows:

Dilutions, from 0.1 to 0.00001, of a twenty-four hour plain broth culture, were made, so that 0.5 c.c. of dilution contained the required amount of culture. The protective sera were also diluted so that one protective unit (0.2 c.c.) was contained in 0.5 c.c. All injections were made intraperitoneally. Three series of mice were treated as follows: series 1 received varying dilutions of the broth culture, called the pathogenicity control; series 2, the same dilutions of culture plus one unit of serum I; series 3, the same dilutions of culture plus one unit of serum II. The animals were kept under observation for a period of five days. On account of its greater delicacy, the protection test is considered more definite than the agglutination.

Table I presents the findings together with a brief description of each case.

TABLE I.

Case No.	Stage, Turban.	Gaffky sputum count.	Description of case.	Prognosis.	Pneumococcus in sputum unwashed.	Pneumococcus in sputum washed.	Virulence.	Remarks.
1	III	IV	Active lesion; recent hemorrhages	Unfavorable	▯	0	.....	2 specimens examined. Patient hemorrhaging at time of collection.
2	III	IV	Progressive; toxic; hydrothorax	Died	IV	IV	0.1 D. 96 0.01 D. 96 0.001 D. 120 0.0001 S. 120 0.00001 D. 96	Patient died 1 mo. after examination.
3	III	VI	Large cavity; no hemoptysis; lesion stationary at present	No improvement to be expected	▯	▯	.....	2 specimens examined.
4	II	IV	Active lesion; toxic	Not good	I	I	0.1 D. 24 0.01 D. 24 0.001 D. 24 0.0001 D. 48 0.00001 D. 48	.....
5	II	V	Recurrent hemoptysis; no cavity; chronic type	Unfavorable	IV	IV	0.1 D. 24 0.01 D. 24 0.001 D. 24 0.0001 D. 48 0.00001 D. 48	.....
6	III	IV	Large cavity; lesion progressive	Bad	▯	▯	.....	2 specimens examined.
7	III	V	Small cavity (old); lesion stationary	Fair	▯	▯	.....	2 specimens examined.
8	II	Neg.	Arrested hemorrhage case	Favorable	▯	0	.....	Friedländer's bacillus recovered.
9	....	Neg.	Bronchiectasis	Unfavorable	0	0	.....	.....
10	II	Neg.	Hemorrhage; bronchiectasis	Favorable	IV	IV	0.1 D. 24 0.01 D. 24 0.001 D. 24 0.0001 D. 72 0.00001 S. 120	.....
11	III	VIII	Cavity; active	Unfavorable	0	▯	.....	2 specimens examined.
12	II	Neg.	Pneumothorax; lesion quiescent	Favorable	0	0	.....	.....

D. = died; S. = survived. The figures represent the number of hours before the death of the animal.

Inulin, bile, and litmus milk were positive on all except strain 47, which was insoluble in bile.

TABLE I.—*Continued.*

Case No.	Stage, Turban.	Gaffky sputum count.	Description of case.	Prognosis.	Pneumococcus in sputum unwashed.	Pneumococcus in sputum washed.	Virulence.	Remarks.
13	....	Neg.	Bronchiectasis; history of previous pneumonia	Unfavorable	III	III	0.1 D. 24 0.01 D. 24 0.001 D. 24 0.0001 D. 36 0.00001 D. 36	2 specimens examined.
14	III	III	Old cavity; no hemoptysis	Bad	0	0	.....	.....
15	....	Neg.	Bronchiectasis; lesion improving	Fair	0	0	.....	2 specimens examined.
16	III	VIII	Large cavity; progressive	Very bad	0	0	.....	.....
17	II	Neg.	Beginning tissue destruction; progressive case	Bad	0	0	.....	.....
18	III	VI	Recent hemorrhage; progressive	Very unfavorable	0	0	.....	2 specimens examined.
19	II	IV	Active lesion	Favorable	IV	IV	0.1 D. 24 0.01 D. 24 0.001 D. 24 0.0001 D. 24 0.00001 D. 24	.....
20	II	VII	Active; progressive	Unfavorable	0	0	.....	3 specimens examined.
21	II	VI	Chronic	Unfavorable	IV	IV	0.1 D. 24 0.01 D. 24 0.001 D. 24 0.0001 D. 72 0.00001 S. 120	.....
22	II	IV	Arrested hemorrhage case	Favorable	0	0	.....	.....
23	II	III	Recurrent hemoptysis	Fair	0	0	.....	.....
24	II	IV	Hemorrhage; active lesion	Favorable	IV	IV	0.1 D. 24 0.01 S. 120 0.001 S. 120 0.0001 S. 120 0.00001 S. 120	Patient hemorrhaging at time of examination.
25	II +	VI	Cavity; hemorrhage	Unfavorable	0	0	.....	2 specimens examined, 2 and 4 dys. after hemorrhage.
26	II	IV	Miliary deposits; hemorrhage	Favorable	0	0	.....	2 specimens examined, 1 and 3 dys. after slight hemorrhage.

TABLE I.—*Continued.*

Case No.	Stage, Turban.	Gaffky sputum count.	Description of case.	Prognosis.	Pneumococcus in sputum unwashed.	Pneumococcus in sputum washed.	Virulence.	Remarks.
27	II	VIII	Active lesion; hemorrhage; pneumonia 18 yrs. ago	Favorable	0	0	.....	.....
28	I	VI	Active lesion	Favorable	0	0	.....	.....
29	III	IV	Lesion stationary; occasional hemorrhage	Unfavorable	0	0	.....	.....
30	III	IX	Cavity; progressive	Unfavorable	IV	IV	0.1 D. 24 0.01 D. 48 0.001 D. 48 0.0001 D. 48 0.00001 D. 72	.....
31	III	IV	Cavity; progressive	Unfavorable	IV	IV	0.1 D. 24 0.01 D. 24 0.001 D. 48	.....
32	....	Neg.	Bronchial asthma; non-tuberculous	Improvement marked	III	Not done	0.1 D. 18 0.01 D. 18 0.001 D. 18 0.0001 D. 36 0.00001 D. 36	.....
33	III	III	Base cavity; progressive	Unfavorable	0	0	.....	.....
34	III	III	Progressive	Very bad	0	0	.....	Gaffky count has been higher.
35	III	V	Large cavity; progressive	Very bad	IV	IV	0.1 D. 24 0.01 D. 24 0.001 D. 48 0.0001 S. 120	Sputum always blood-streaked.
36	III	IV	Cavity; progressive	Bad	0	0	.....	.....
37	III	IV	Cavity; progressive	Bad	IV	IV	0.1 D. 24 0.01 D. 24 0.001 D. 72 0.0001 D. 96 0.00001 S. 120	Presumptive evidence of intestinal tuberculosis.
38	III	V	Large cavity; progressive	Bad	0	0	.....	.....
39	II	Neg.	Recent large hemorrhage; lesion slowly progressive	Unfavorable	0	0	.....	.....
40	III	III	Cavity; progressive	Unfavorable	0	0	.....	.....
41	III	II	Old cavity; chronic case	Unfavorable	0	0	.....	.....

TABLE I.—*Concluded.*

Case No.	Stage, Turban.	Gaffky sputum count.	Description of case.	Prognosis.	Pneumococcus in sputum unwashed.	Pneumococcus in sputum washed.	Virulence.	Remarks.
42	....	Neg.	Cavity quiescent; possibly bronchiectasis	Very favorable	IV	1st specimen III 2d specimen IV	0.1 D. 24 0.01 D. 24 0.001 D. 48	Virulence done on unwashed strain.
43	II	VI	Chronic case, 15 yrs.' duration; pneumonia, Jan., 1914	Favorable	I	I	0.1 D. 24 0.01 D. 24 0.001 D. 24 0.0001 D. 24 0.00001 D. 24	On protection against serum I, all mice died on 5th dy. Agglutination typical; immediate.
44	II	III	Lesion stationary	Favorable	0	0	.....	.....
45	I	III	Occasional hemorrhage; condition good	Favorable	III	IV	0.1 D. 24 0.01 D. 24 0.001 D. 24 0.0001 D. 24 0.00001 D. 48	Virulence is recorded for unwashed strain. Virulence not done on washed strain.
46	II	V	Condition good	Good	0	0	.....	.....
47	II	III	Lesion stationary; improving	Good	IV	IV	0.1 D. 24 0.01 D. 24 0.001 D. 72 0.0001 D. 48 0.00001 D. 24	This strain not soluble in bile.
48	....	Neg.	Bronchial asthma; tuberculosis questionable	Good	IV	Not done	0.1 D. 24 0.01 D. 24 0.001 D. 24 0.0001 D. 24 0.00001 D. 24	Washed specimen not done. Patient had gone home.
49	III	III	Old cavity; slowly progressive	Unfavorable	IV	IV	0.1 D. 24 0.01 D. 24 0.001 D. 24 0.0001 D. 24 0.00001 S. 120	.....
50	....	Neg.	Bronchiectasis	Favorable	IV	IV	0.1 D. 24 0.01 D. 24 0.001 D. 24 0.0001 D. 72 0.00001 D. 96	.....

In the fifty cases tabulated pneumococci were isolated in twenty only, or 40 per cent. With the exception of two cases in which no washed specimen was examined, there were only two differences in the findings on the washed and unwashed specimens. The percentage of positive findings is low compared with the figures given for pneumococci in normal mouths by Longcope and Fox,[4] Buerger,[5] and Wadsworth.[6] This may be due to the favorable influence of sanatorium treatment. On the other hand, the percentage figure is considerably higher than that ordinarily reported for this type of organism as a secondary invader in pulmonary tuberculosis.[7] There are two possible explanations of the higher figure in this instance; first, the application of the more exact technique, and, second, the fact that, as previously mentioned, only cases were selected for examination which showed a progressive lung condition.

Of the forty-three cases of tuberculosis, pneumococci were found in fifteen (34.9 per cent.). The positive cases included six out of the eighteen with definite cavity formation, and four out of the eleven hemorrhage cases. There seems to be no definite correlation between the type of case and the presence of pneumococci in the sputa. Positive results were obtained in three out of five cases of bronchiectasis and in both cases of asthma.

By classifying the pneumococci isolated according to groups, it will be noted that, of the twenty positive cases, group IV organisms were found in fifteen (75 per cent.), group III in three (15 per cent.), and group I in two (10 per cent.), when case 43 is included. No organisms of group II were isolated. Dochez and Avery[8] have

[4] Longcope, W. T., and Fox, W. W., A Comparative Study of Pneumococci and Streptococci from the Mouths of Healthy Individuals and from Pathological Conditions, Report of the Medical Commission for the Investigation of Acute Respiratory Diseases, Department of Health of the City of New York, *Jour. Exper. Med.*, 1905, vii, 430.

[5] Buerger, L., Studies of the Pneumococcus and Allied Organisms with Reference to Their Occurrence in the Human Mouth, Report of the Medical Commission for the Investigation of Acute Respiratory Diseases, Department of Health of the City of New York, *Jour. Exper. Med.*, 1905, vii, 497.

[6] Wadsworth, A., Experimental Studies on the Etiology of Acute Pneumonitis, *Am. Jour. Med. Sc.*, 1904, cxxvii, 851.

[7] Avery, O. T., and Lyall, H. W., Concerning Secondary Infection in Pulmonary Tuberculosis, *Jour. Med. Research*, 1913, xxviii, 111.

[8] Dochez, A. R., and Avery, O. T., Varieties of Pneumococcus and Their Relation to Lobar Pneumonia, *Jour. Exper. Med.*, 1915, xxi, 114.

shown that the organisms usually present in the mouths of normal persons belong in group IV, while pneumococci of the so called fixed types, especially those of groups I and II, occur with great infrequency, and only under such conditions as apparently to justify considering persons who harbor them as carriers of infection. The study of this small number of cases of tuberculosis indicates that the type of pneumococci present is usually that found in the normal mouth, and that the fixed types present in the majority of cases of acute lobar pneumonia occur only infrequently. Further study will be required to show whether the fixed types occur more commonly in the mouth and sputum of patients with tuberculosis than they do in the mouths of normal persons. It must be remembered that the organisms placed in group III are so classified on certain morphological and cultural characters. Whether all the organisms placed in this group are identical from the standpoint of infection and immunity is not yet certain.

It is of significance that, among the twenty organisms studied, only two belonged in groups I and II. One of the patients harboring such an organism had had an attack of pneumonia six months previously. While the evidence obtained does not exclude the possibility that transformation or mutation of type may occur in cases with pathological lung lesions, the occasional occurrence of specialized types in these cases is probably to be explained on other grounds.

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FURTHER INVESTIGATIONS ON THE MODE OF ACTION OF SUBSTANCES INHIBITING TUMOR GROWTH AND ON IMMUNIZATION AGAINST THESE SUBSTANCES.*

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The results which we obtained in our previous investigations on the mode of action of substances inhibiting tumor growth[1] were based partly on statistical methods; we therefore hesitated in our previous communications to pronounce our results as definite. The importance of these results, however, seemed to us to call for further experiments which should test the validity of our conclusions and extend the experimental basis on which they rested. In order to confirm these experiments we used a large number of mice, 604 of which lived to the end of the experiments and are included in our report.

THE SPECIFICITY OF THE IMMUNIZING ACTION OF INJECTIONS OF HIRUDIN AND COLLOIDAL COPPER.

In our previous paper[2] we concluded from the results of experiments carried out with the first method that the immunity against the influence of colloidal copper and hirudin in inhibiting tumor growth conferred through several injections of these substances, is, in the main, specific; that injections of colloidal copper immunize principally against the effects of colloidal copper and not, or only to a slight extent, against those of hirudin, and that hirudin immunizes principally against the effects of hirudin.

It was much to be desired that these conclusions should be further tested by means of the more exact second method. We made, there-

* Received for publication, October 28, 1914.

[1] Fleisher, M. S., Vera, M., and Loeb, L., *Jour. Exper. Med.*, 1914, xx, 522.

[2] Fleisher, Vera, and Loeb, *loc. cit.*

fore, two additional experiments, the results of which are given in table I in horizontal columns 3 and 4.

TABLE I.

	No. of mice.	α	β	γ	δ	a	c	d	f	Horizontal column.
E–1 Controls	22	—	—	80%	—	—	4 81%	10 83%	8 75%	1
E–1 Copper controls: 9th–13th dy.	51	66%	55%	58%	60%	8 96%	14 55%	18 59%	11 20%	1
E–1 Immunized: 2d–6th dy. Copper: 9th–13th dy.	52	98%	87%	88%	91%	8 118%	12 84%	19 77%	13 74%	1
E–2 Controls	18	138%	144%	136%	139%	3 151%	2 125%	7 136%	6 127%	2
E–2 Copper controls: 9th–13th dy.	64	87%	81%	78%	83%	7 72%	9 76%	21 74%	27 92%	2
E–2 Copper series: transplanted 9th–13th dy.	70	138%	135%	134%	136%	10 121%	20 117%	24 147%	16 150%	2
F Controls	31	158%	158%	167%	161%	7 176%	13 134%	7 170%	4 188%	3
F Hirudin: 2d–6th dy. Hirudin: 9th–13th dy.	72	157%	149%	152%	153%	11 173%	19 148%	26 143%	16 145%	3
F Hirudin: 2d–6th dy. Copper: 9th–13th dy.	72	123%	117%	122%	120%	11 163%	24 108%	24 112%	13 105%	3
G Controls	28	141%	149%	150%	147%	—	6 153%	8 149%	14 147%	4
G Copper: 2d–6th dy. Hirudin: 9th–13th dy.	61	76%	75%	73%	74%	—	8 59%	23 98%	30 62%	4
G Copper: 2d–6th dy. Copper: 9th–13th dy.	63	150%	136%	147%	144%	—	6 175%	22 134%	35 131%	4

α Percentage of increase on comparing the sum of the original weight of all tumors (calculated) with the sum of the end weight (weighed).

β Multiply the average percentage of increase of each class (a, c, d, f) by the number of mice in that class, add the figures thus obtained, and divide the sum by the total number of mice used in that series.

γ Average percentage of increase of the various classes; add the percentage increases in each class and divide by the number of classes.

δ Average of the three preceding averages.

In the tables the series in each column which have the same mark, for instance A1 and B1, represent experiments done under approximately the same conditions and at the same time.

The figures above and to the left of the main figures indicate the number of mice used in each class.

In the experiment recorded in column 3, 175 mice lived until the end of the experiment; 31 served as controls; 144 mice received from the second to the sixth day a daily injection of hirudin, each animal thus receiving four preliminary (preparatory) injections. Of these 144 mice, one-half received from the ninth to the thirteenth day injections of hirudin, while the second half received colloidal copper. The same doses of these substances were used as in our previous experiments.[3] According to the weight of the mouse tumors on the ninth day, we divided them into the same four lots (a, c, d, f) as in the previous experiments. Such a division is necessary because the rapidity of growth of a tumor in a given period is, among other factors, as a rule, a function of its weight. In these experiments the difference in the various classes happens to be slight. The increase in weight in the different classes from the ninth to the thirteenth day is given in the vertical columns. In the vertical columns, α, β, γ, δ, the average of the increase in weight of the tumors of all the mice treated in the same manner is given, calculated according to the four different procedures which have been described in our previous paper.[4] We find that the mice which had been treated with injections of hirudin from the second to the sixth day were only slightly affected by the second set of injections of hirudin. The figures showing the percentage increase in weight from the ninth to the thirteenth day, during which period the animals received injections of hirudin, were only slightly less than those of the controls; while, on the other hand, the mice which received during this same period injections of colloidal copper were noticeably inhibited in their tumor growth. We may therefore conclude that while the preliminary injections of hirudin immunized the animals almost completely, or at least very markedly, against the effect of later injections of hirudin, they did not exert such an effect towards

[3] Fleisher, Vera, and Loeb, *loc. cit.*

[4] Fleisher, Vera, and Loeb, *loc. cit.*

later injections of colloidal copper, in so far as the immunizing effect of preliminary injections of hirudin is a specific one. This conclusion is confirmed by the result of the experiment in horizontal column 4. 124 animals received from the second to the sixth day after inoculation daily intravenous injections of colloidal copper; 63 of these mice received again colloidal copper from the ninth to the thirteenth day; while 61 mice received hirudin from the ninth to the thirteenth day. In the first lot, immunized against the action of colloidal copper, the colloidal copper exerted no, or only a very weak inhibiting action on the tumors, which grew therefore almost as well as in the controls; while in the second lot, which was again immunized against colloidal copper, but not against hirudin, injections of hirudin exerted a marked inhibiting action on tumor growth. This experiment at the same time proves that the hirudin used in the experiment recorded in horizontal column 3 would have been sufficiently strong to exert an inhibiting effect in the latter experiment, provided that the mice had not been immunized against the inhibiting action of hirudin. We may, therefore, on the basis of these, and of our previously recorded experiments, conclude that the immunizing action of preliminary injections of hirudin and colloidal copper is specific.

THE IMMUNIZATION OF TUMOR CELLS AGAINST THE EFFECT OF COLLOIDAL COPPER AND THE TRANSMISSION OF THIS IMMUNITY TO SUCCEEDING CELL GENERATIONS.

Further experiments were carried out in order to test and, if possible, to confirm our previous results, which indicated that the immunity produced against substances inhibiting tumor growth resides partly in the tumor cells and is transmitted to succeeeding generations of tumor cells. In horizontal column 2, table I, an additional experiment of this kind is recorded. 152 animals lived to the end of the experiment; 18 were controls, not injected animals; all the others received from the ninth to the thirteenth day injections of colloidal copper; 70 of these 134 mice had been inoculated with pieces from tumors grown in mice which had received injections of colloidal copper from the second to the sixth day and from the ninth to the thirteenth day. An immunity had therefore presumably been

established in the tumor cells against the effect of colloidal copper. That this is the case is shown in horizontal column 2. In the last lot of 70 mice, the tumors grew approximately as well as in the controls, despite the injections of colloidal copper which these 70 mice received, while in the 64 mice with non-immunized tumors, the tumor growth was inhibited to the usual extent through the injections of colloidal copper given from the ninth to the thirteenth day. In addition we carried out another experiment in which is compared the effect of colloidal copper on tumors in 52 animals which had been immunized against the effect of colloidal copper through preceding injections with this substance, given from the second to the sixth day, and in 51 animals which had not received a series of preliminary injections. We see that in the immunized animals the tumors grew about as well as in the non-injected controls, while in the non-immunized animals the tumor growth was inhibited through colloidal copper. These results again confirm our previous conclusions.

THE INEFFICACY OF INJECTIONS OF COLLOIDAL COPPER AND OF HIRUDIN ON VERY YOUNG TUMORS.

As we have observed previously, the weight of a tumor on the ninth day after transplantation usually enters as a factor into the set of conditions which determine the increase in weight of the tumors in the following four days. In order to rule out a possible influence of this factor on the results of our experiments, we had to compare on the ninth day the average weight of the various lots of tumors, arranged in the various classes. Tumors of smaller weight usually show in the following period from the ninth to the thirteenth day a greater increase in weight than tumors of larger weight. We must examine whether the differences in the weight of the various lots correspond and can be held responsible for the differences observed in weight increase from the ninth to the thirteenth day in immunized and non-immunized mice. Such a table should also give evidence of a possible inhibiting effect of intravenous injections of colloidal copper or of hirudin given from the second to the sixth day. If such an inhibiting effect should exist, the average weight of tumors on the ninth day in mice which received these preliminary

TABLE II.

Weight of Various Classes of Tumors at Nine Days.

Column 1. Normal uninjected controls.				Column 2. Mice injected with colloidal copper 9th–13th dy.				Column 3. Mice injected with colloidal copper 2d–6th and 9th–13th dys.				Column 4. Mice inoculated with tumor from mice injected with colloidal copper. Injected with colloidal copper 9th–13th dy.			
a	c	d	f	a	c	d	f	a	c	d	f	a	c	d	f
H				A–1								A–1			
					5	9	10						18	12	21
				—	286	176	58					—	247	173	51
D				A–2				A–2				A–2			
4	8	35	36		6	5	23		5	7	6	—			
367	240	158	92	—	283	198	68	—	270	162	87				
B–1				B–2				B–1							
1	6	6	6	17	30	8	4	12	26	14	8				
350	271	180	91	462	281	177	106	466	291	186	92				
B–2				B–2								B–2			
10	6	5	18	10	14	12	13					9	22	18	13
525	291	137	72	446	297	156	92					421	280	160	60
C–1				C–1				C–1							
3	12	3		14	15	14	15	4	16	19	16				
532	276	190	—	439	293	162	87	447	291	180	84				
C–2												C–2			
		15	35	8	20	15	23					6	22	22	18
—	—	143	68	476	284	156	73					411	292	159	101
E–1				E–1				E–1							
	4	10	8	8	14	18	11	8	12	19	13				
—	308	179	96	434	264	176	94	380	265	162	92				
E–2				E–2								E–2			
3	2	7	6	7	9	21	27					10	20	24	16
426	285	166	73	413	277	164	85					426	292	167	94
Controls:				F Hirudin-copper or		G Copper-copper		F Hirudin-hirudin or		G Copper-hirudin					
F Specimen 1				F				F							
7	13	7	4	11	24	24	13	11	19	26	16				
431	294	180	110	437	294	185	103	415	293	175	104				
G Specimen 2				G				G							
6	8	14			6	22	35		8	23	30				
—	267	153	94	—	263	149	95		255	147	94				

The letters and numbers of each group in table II correspond to the designations in table I of this paper and table I of our previous paper.[5] By comparing this table with the two other tables, it will therefore be possible to determine the increase in weight from the ninth to the thirteenth day of the tumors of the various classes of mice.

[5] Fleisher, Vera, and Loeb, *loc. cit.*

injections should be less. We notice that the animals which received injections of colloidal copper or of hirudin from the second to the sixth day had on the ninth day tumors of approximately the same weight as mice without such injections. In column 3, table II, is given the average weight of the tumors of the various lots of mice in classes, a, c, d, f, according to the weight limits fixed in our previous paper. We see that there is no noticeable difference in the average weight of the different classes of mice, if we compare columns 3 and 2. In the latter is the weight of the tumors of mice which had not received the preliminary injections. In the last two horizontal columns (F and G) we can compare the weight on the ninth day of tumors of non-injected mice (vertical column 1) with those having received hirudin (F vertical column, Nos. 2 and 3) and colloidal copper (G vertical column, Nos. 2 and 3) from the second to the sixth day. We see that on the whole there is no marked difference in the weight of the tumors of the different lots of mice.

In vertical column 4 we find a few classes of tumors with somewhat smaller weight; namely, c and f in horizontal column A^1, and in group F in horizontal column B^2. All others are approximately normal. If we analyze the figures indicating the increase in weight of the tumors from the ninth to the thirteenth day of mice inoculated with immunized tumor material, consulting for this purpose the increase of weight of these tumors given in table I of our previous paper[6] and in table I of this paper, we see that our conclusion as to the lack of inhibition in the growth of tumors of these lots of mice is not dependent on these few classes, but is noticeable in other classes as well. We may therefore conclude that the immunization which we observed in mice having received a preliminary set of injections, either from the second to the sixth day after inoculation, or in the previous generation of mice, is real and is not due merely to the difference in weight of the tumors on the ninth day. We may furthermore conclude that injections of colloidal copper or hirudin at an early stage (from the second to the sixth day) after transplantation do not inhibit tumor growth to a noticeable extent.

[6] Fleisher, Vera, and Loeb, *loc. cit.*

THE EFFECT OF A COMBINATION OF HIRUDIN AND COLLOIDAL COPPER ON TUMOR GROWTH.

In our previous paper we reported that combinations of colloidal copper and hirudin and of colloidal copper and nucleoproteid were much more effective than either of these substances alone, and that these combinations not only caused an inhibition of the tumor growth but also retrogression of a considerable number of tumors; we also found that the combination of colloidal copper and hirudin was more toxic than either substance given alone. In order to confirm this result we carried out an additional experiment of which table III gives the results.

TABLE III.

Substance injected.	Dose.	Number of tumors and mortality.	Result.
Combination of hirudin and copper	0.0125 to 1.25 mg. 0.25 c.c.	27 tumors; mortality 29% (8 died)	4 grew well, 1a, 1c, 2d; 15%. 8 were retarded, 2a, 3c, 3d; 30%. 15 retrogressed, 3a, 6c, 2d, 4f; 55%.
Controls	.....	30 tumors	1 did not grow, 1c; 3%. 29 grew well, 7a, 12c, 7d, 3f; 97%.
Hirudin	0.125 to 1.25 mg.	60 tumors; mortality 15% (9 died)	18 grew well; 30%. 32 were retarded; 53%. 2 retrogressed; 3%. 10 doubtful; 17%.

In the results we state the number of tumors in each class (a, c, d, f) of tumors.

Mice were injected with a mixture of 0.25 of a cubic centimeter of colloidal copper and 0.0125 of a milligram to 1.25 milligrams of hirudin, the smaller dose of hirudin being injected first, and the dose being gradually increased in the course of the following injections; while in the mice injected with hirudin alone 30 per cent. of the tumors grew normally, 17 per cent. were doubtful, 53 per cent. were retarded, and only 3 per cent. retrogressed (the latter being in this case included among the non-growing tumors); only 15 per cent. of tumors grew normally among the mice treated with the combination of hirudin and colloidal copper; 30 per cent. were retarded, and 55 per cent. retrogressed. Among this latter lot of mice there had been

a mortality of 29 per cent. (only the mice living to the end of the experiment being included in the list); among the mice injected with hirudin only, the mortality was smaller; *viz.*, 15 per cent. We see, therefore, that the combination of hirudin and colloidal copper is much more effective than either substance given alone.

These additional investigations confirm, therefore, our previous experiments, and we may now with still greater certainty draw the general conclusions which we mentioned in our previous paper on immunization against the action of substances inhibiting tumor growth.

SUMMARY.

Our later investigations confirm our previous results.

1. It is possible to increase markedly the effect of substances inhibiting tumor growth by using certain combinations of these substances which, when given alone, have some effect on tumor growth.

2. Immunity acquired against the effect of these substances depends partly upon an active immunization of the tumor cells themselves against the action of these substances, and this immunity is transmitted to the following generations of tumor cells.

3. The immunity against the substances inhibiting tumor growth is, as far as we have investigated the problem, specific.

4. Our later experiments provide a more secure basis for the additional and more general conclusions which we mentioned tentatively in our previous paper.

A COMPARISON OF AUTOPLASTIC AND HOMEOPLASTIC TRANSPLANTATION OF THYROID TISSUE IN THE GUINEA PIG.*

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It is our purpose in this work, undertaken at the suggestion and carried out with the aid of Dr. Leo Loeb, to trace the fate of the thyroid gland after homeoplastic transplantation, and to compare it with the behavior of this tissue after autoplastic transplantation.

The vast literature of thyroid transplantation relates chiefly to autoplastic transplantation. A few brief statements on homeotransplantation are made by Cristiani (1). An accurate comparative analysis of the difference in the growth of auto- and homeoplastically transplanted thyroid tissue does, however, not exist. A communication, published recently by Leischner and Köhler (2) mentions only the disappearance and resorption of the homeograft, without giving histological descriptions.

The findings of the various authors, as far as they concern the fate of thyroid tissue after autotransplantation, agree in general. Von Eiselsberg's (3) successful implantation of cat thyroid (1892), with an histological description, was in all points confirmed by Sultan (1898) (4). The work of Enderlen (1898) (5) deals with the successful implantation of the thyroid as an established fact and takes up the further question, whether the transplanted gland continues to exert its normal function, and whether it produces and eliminates a sufficient quantity of colloid. Enderlen concludes that while the transplanted gland produces colloid, it does so in insufficient quantity, and that the elimination of the colloid is insufficient.

The same question concerning the secretion of colloid on the part of the transplanted gland was raised again in 1906 by Payer (6), who transplanted thyroids of dogs, cats, rabbits, and guinea pigs into the lower pole of the spleen, which he considers favorable soil; he finds an extensive elimination of colloid, taking the presence of colloid in the lymphatic vessels of the spleen as proof of this elimination.

Salzer (7), who transplanted thyroid into the abdominal musculature, considers this tissue likewise favorable soil for the absorption of colloid. He states that in some places the muscle tissue directly surrounds the thyroid vesicles; that in other places, however, the implanted gland is surrounded by a very fine connective tissue capsule; at no place was Salzer able to find a more or less dense connective tissue layer surrounding the implanted graft.

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Numerous papers deal with technical questions in addition to the histological description; for instance, the soil most favorable for transplantation. Thus Cristiani writes exclusively on questions of technique (over thirty papers, 1894–1906); he discusses especially the size of the transplanted piece and the importance of the capsule for successful transplantation.

In his first work Cristiani used the same technique for subcutaneous or intraperitoneal transplantation as his predecessors; in his later work he gives preference to subcutaneous transplantation into the ear of the rabbit, as this method permits better observation of the size, transparency, and vascularization of the graft. Salzer prefers intramuscular transplantation into the abdominal wall; he considers this method preferable to the one recommended by Serman and Kocher (1908) (8) of transplantation into the metaphysis of the femur.

Von Eiselsberg (3) already gives as important factors for successful transplantation asepsis and rapid transfer (within ten seconds) of the still warm, living tissue. Cristiani found that exposure of the pieces to the air for ten seconds does not injure the tissue, but that a longer exposure markedly diminishes its vitality. Cristiani successfully carried out autotransplantations in mammals, birds, reptiles, and amphibia; in fishes he did not succeed on account of technical difficulties. According to Cristiani, the thyroid continues to live and to grow after autoplastic transplantation; even the transplanted thyroid, if transplanted a second time, grows again, provided the transplantation had not been undertaken at too early a date.

Cristiani found, furthermore, a remarkable power of resistance of the transplanted thyroid against different infections with various microörganisms (*B. coli*, streptococcus, and staphylococcus); even different substances, such as turpentine, usually causing necrosis, did not injure the transplanted graft.

In this laboratory successful transplantations of the thyroid were obtained by Carroll Smith (9) in connection with a different problem. He describes, however, only the autoplastically transplanted pieces, without reference to the behavior of the corresponding homeografts.

METHOD.

In our experiments we used guinea pigs, working always on two animals. The neck and abdominal region of the animals are shaved and cleaned with alcohol and a solution of mercury bichloride, then covered with sterile towels soaked in the mercury bichloride solution, and the animals are narcotized with ether. An incision 1 to 2 cm. in length is made in the abdominal region. Into this incision an instrument is introduced and narrow pockets are made on the right and left sides between the skin and subcutaneous tissue or the abdominal musculature, at a distance of about 3 to 4 cm. from the point of incision.

Our series includes over 75 animals with more than 140 grafts. Pieces of the remaining part of the thyroid gland were examined microscopically. The animals were killed with chloroform at intervals of 24, 36, 48, and 72 hours, and 4, 5, 7, 9, 11, 12, 13, 14, 16, 17, 19, 22, 26, 27, 29, 31, 33, 40, 43, 47, and 52 days after the trans-

plantation; the grafts and the parts of the remaining thyroid lobe were preserved in Zenker's fluid and cut serially.

DESCRIPTION OF THE TRANSPLANTS.

Transplanted tissue removed 24, 36, and 48 hours after transplantation was necrotic, with the exception of a narrow peripheral zone. Towards the periphery numerous widely dilated capillaries with an occasional fibroblast between them are seen, penetrating into the central necrotic area. The peripheral zone consists largely of more or less well preserved follicles with epithelium and colloid, which appears retracted and lying free in the lumen of the follicle. The epithelium is flat or cuboidal; its nuclei are vesicular and rather dark. Mitoses are absent. In the surrounding tissue isolated fibroblasts, lymphocytes, and polynuclear leucocytes are seen. The grafts lie free in the pockets. There is at this period no difference between the auto- and homeograft.

After 72 Hours.—An extensive central necrotic area is still present, but it is now divided into smaller parts by a wide network of capillaries. The number of chromatin particles and pycnotic nuclei in the necrotic material is diminished, while the fibroblasts, polynuclear leucocytes, and lymphocytes are increased. The peripheral living follicular zone has become larger and consists of two to three rows of follicles, lined with high cuboidal epithelium, containing light vesicular nuclei, which already show some, although not yet numerous mitotic figures. Colloid is present in the majority of the follicles. At several places fibroblasts from the periphery are invading the spaces between the follicles; they originate from the host tissue and are very numerous in the tissue surrounding the grafts; mitoses are quite frequent in these cells. There is not yet any distinct difference between auto- and homeotransplant, although the blood vessel supply in one homeograft is smaller.

After 4 Days.—A central necrotic area is still present, but the detritus has much decreased in quantity; the chromatin particles and pycnotic nuclei are less numerous. Dilated capillaries, accompanied by fibroblasts, containing mitoses, are numerous in the necrotic area. Lymphocytes and polynuclear leucocytes are met only here and there. Towards the periphery we meet larger follicles with flat epithelium, with small, moderately light nuclei and solid colloid. Some small new follicles are found in the periphery, which are either entirely isolated or united in groups of two or three. Their epithelium is high cuboidal; the nuclei are large and vesicular, some containing mitoses. Colloid is absent in the smaller follicles. A few isolated, solid cell nests, consisting of six to ten circularly arranged cells, are also seen; several show already the beginning of a central lumen. Numerous fibroblasts, frequently with mitoses, are penetrating from the surrounding tissue in several directions into the transplanted tissue. Lymphocytes and polynuclear leucocytes are infrequent. Auto- and homeotransplant are identical at this period.

After 5 Days.—Both grafts are partly and only very loosely adherent to the surrounding host tissue; such adhesions become more and more marked in the following days and are very strong after the 12th day; after this stage the removal of the host tissue together with the graft becomes therefore necessary.

After 5 days what was formerly the central necrotic part is still persisting in the autograft, but it is now much smaller than in the earlier stages and is represented chiefly by dilated capillaries surrounded by numerous fibroblasts which have invaded this area. The number of colloid-bearing follicles has increased very much and this follicular zone occupies now a relatively much larger part of the section. Small solid cell areas and short tortuous tubules with high cylindrical epithelium and with very closely arranged nuclei are seen more frequently at this period; they are situated either in the periphery or further towards the center; these cell tubules show frequently evagination into the surrounding tissue as well as papillary protrusions into the lumen. Various stages of the separation of small round follicles from these tubules can be seen. Mitotic figures have become numerous; frequently as many as eight can be seen in one field. Colloid is absent in the small follicles. Lymphocytes and polynuclear leucocytes are seldom seen; on the other hand, fibroblasts are frequent, especially in the peripheral parts and in the surrounding tissue, from where they invade the grafts arranged in bundles and in different directions.

In a corresponding homeograft the same conditions prevail in the periphery, but the larger part of the section is occupied by an extensive area of extravasated blood. Another homeograft 5 days old, however, differs already from the corresponding autograft. (1) In the former a much larger number of lymphocytes is present, which protrude into and between the follicles and begin to cause the destruction of the follicles. In some places they form small accumulations; they are seen in the larger blood vessels and in the connective tissue from where they penetrate towards the follicles. (2) The number of fibroblasts is greater in the homeograft, and their arrangement is at several places less loose than in the autograft; in dense bundles they invade the tissue between the follicles, compressing several of them. But this destruction is as yet only very slight; there is merely an indication of it present. In one homeograft are seen even especially long tortuous cell cords, consisting of two rows of closely adjoining cylindrical cells which in some places separate to form a narrow lumen. In oblique sections they appear as isolated small cell nests or groups of cell nests, with numerous mitoses. In cross-sections they represent oval or round follicles with cylindrical epithelium.

After 7 Days.—The central part of the autograft, consisting in some places chiefly of blood vessels, is still distinct, but much smaller than at the earlier stages. The number of fibroblasts in this part is also markedly increased, but their arrangement is very loose. Lymphocytes are rare and found chiefly in the blood vessels. The old transplanted blood vessels are nearly entirely occluded through the proliferation of cells derived from the blood vessel walls. The central connective tissue contains numerous brownish yellow blood pigment cells. The number of follicles with high cuboidal epithelium is large; they are mostly round or oval and not compressed. We find also tubular epithelial structures with irregular evaginations and some mitoses. Several follicles contain blood and desquamated cells. The graft is surrounded by a rather dense connective tissue.

There is after 7 days a distinct difference between the homeo- and autograft. In the former the number of follicles and the amount of colloid is markedly decreased; the preserved follicles, which are present only at the periphery, are compressed by connective tissue, which invades the spaces between them; the follicles are frequently smaller and flatter than in the autograft and contain more rarely colloid, but more frequently desquamated cells. Usually the epithelium is of normal height and may also contain mitoses. As in the grafts 5 days old, there are numerous lymphocytes in the connective tissue and around the follicles, into which they migrate, causing their destruction. In addition, we find between the individual follicles a larger number of densely arranged connective tissue cells with mitoses in the process of producing fibrous tissue. The greater part of the graft, especially its central parts, consists of a densely built connective tissue, which in some places appears hyaline; it contains also lymphocytes and traces of destroyed or compressed follicles, which appear as narrow clefts, containing several desquamated cells. The graft is surrounded by a dense connective tissue with numerous mitoses in the fibroblasts.

After 9 Days.—The difference between auto- and homeograft is distinct. In the autograft a larger number of well preserved colloid-containing follicles, the epithelial cells of which contain numerous mitoses, is present; these follicles are separated by a thin layer of connective tissue. Lymphocytes do not invade the epithelium. The central parts of the pieces consist of loosely arranged connective tissue cells with light vesicular nuclei; there are also present numerous brown blood pigment cells. Isolated small or large solid cell nests, as well as small groups of small empty follicles, are frequent in the central areas. In some places the cell nests are strongly developed, and in such cases a large number of mitoses may be seen in the epithelial cells; as, for instance, eight to fifteen in one field. Lymphocytes are rarely present and usually in small isolated nests. Polynuclear leucocytes are occasionally seen in the larger blood vessels. The graft is surrounded by a moderately dense connective tissue.

In the homeograft only isolated follicles, separated and strongly compressed by surrounding connective tissue bands, are seen. They are found mostly in the periphery. The larger part of the follicles in the homeograft is destroyed by lymphocytes; this destruction can be followed especially distinctly in the case of the isolated central follicles; these are surrounded by numerous lymphocytes, which invade the follicles, and penetrate between the epithelial cells, destroying them. The preserved follicles have a flat epithelium with compressed nuclei, which rarely contain mitoses. A few follicles contain solid colloid. Large areas of the sections are occupied by lymphocytes, which fill also the larger blood vessels. The transplanted parathyroid shows the same destruction through lymphocytes as the thyroid tissue. The central parts of the graft consist partly of dense masses of fibroblasts, with distinct nuclei, which in some places form a fibrous, nearly hyaline tissue. In the center also lymphocytes are numerous, forming rather large accumulations and filling the larger blood vessels. The original transplanted blood vessels show a totally obliterated lumen and a remarkably thickened wall, in the cells of which mitoses may be visible. Rarely an isolated follicle with cuboidal epithelium, occasionally infiltrated by lymphocytes, is seen in the central parts. Some follicles do not contain any colloid, but only desquamated cells. Blood vessels are rarely seen. The graft is surrounded

by densely built connective tissue, which penetrates widely into the interior of the section.

After 11 Days.—In the autograft the number of follicles with cuboidal epithelium is increasing; they are now seen not only in the peripheral, but also in the central zones, either isolated or combined into small groups. Formation of papillæ and invagination and evagination of the epithelium are frequent in these follicles; furthermore, small new follicles with numerous mitoses and without colloid seem to develop from the larger tubes. Numerous follicles are filled with red corpuscles, with lymphocytes and polynuclear leucocytes among them. Between the follicles are fibroblasts, but their number is small and they are loosely arranged; they contain occasionally a mitotic figure. Lymphocytes are absent; polynuclear leucocytes are found only in a very limited number; but brownish blood pigment cells are frequent. The blood vessel supply of the graft is extensive. The central former necrotic area is now much smaller and consists of a net of capillaries, filled with red blood cells; between the capillaries are numerous isolated fibroblasts; several lymphocytes can also be seen, but polynuclear leucocytes are more frequent. Near the thyroid, and separated from it only by a narrow layer of loosely arranged fibroblasts, parathyroid tissue is seen, which appears entirely normal, with the exception of the presence of a few fibroblasts. The entire graft is surrounded by a ring of densely arranged fibroblasts, which in several places are compressing each other and appear narrow; in other places the arrangement is looser, the nuclei are round and vesicular, with two to three mitoses in a field. Lymphocytes and polynuclear leucocytes are rare.

After 11 days the difference between auto- and homeograft is very distinct; in the latter the number of preserved follicles is diminished; the follicles that are left are smaller; the epithelium is often flat, with elongated nuclei, which rarely show mitoses. Colloid is present in the majority of follicles, but it is retracted, solid, and without vacuoles. Lymphocytes and polynuclear leucocytes are not seen frequently. In several places fibroblasts are invading in loose bundles the graft between the follicles. Brown blood pigment cells are frequent. The larger central part of the graft consists of a granular mass stained reddish with eosin—the remnants of the necrotic parts of the transplanted tissue and of fibroblasts which have penetrated into it. Lymphocytes and leucocytes are absent. The blood supply is limited. Numerous phagocytic connective tissue cells are present in the necrotic material.

After 12 Days.—Both grafts are moderately firmly adherent to the adjacent tissue. Microscopically the autograft appears similar to the one excised 11 days after transplantation. There are seen numerous round and oval follicles, located mostly at the periphery. While colloid is filling the majority of the follicles, several are empty and others contain desquamated cells with pycnotic nuclei. The larger part of the graft is occupied by a loosely built connective tissue, in which is occasionally embedded a follicle with high cuboidal epithelium. Several foreign body giant-cells are present. Polynuclear leucocytes are here only rarely seen, but they are more frequent than lymphocytes. The graft is now, and usually also at later periods, surrounded by a densely built connective tissue. The homeograft after 12 days is very different. Only a small number of follicles with insignificant remnants of colloid is present. They are usually found in the periphery and their epithelium is partly preserved, partly desquamated, and

with a protoplasm which is often vacuolar; the nuclei are vesicular and frequently indented. Most of the follicles are markedly compressed by connective tissue fibers and show only a narrow lumen. Frequently lymphocytes surround the follicles, invading and destroying the epithelium. The preserved follicles are separated from each other by dense hyaline connective tissue, in which remnants of cell detritus can be seen; a small number of lymphocytes and polynuclear leucocytes are also present. The graft is, as in the future periods, surrounded by a dense connective tissue, containing many lymphocytes.

After 13 Days.—The number of follicles filled with colloid is increased in the autograft; they are lined with cuboidal epithelium, containing vesicular nuclei, with occasional mitoses. In general the number of mitoses is now smaller than in an earlier period; namely, up to the 9th day. Occasionally small solid cell areas are seen. The numerous follicles are located here as in the normal gland close to each other, divided by scanty connective tissue. Lymphocytes and polynuclear leucocytes are absent. The central part of the graft, which is without follicles, is relatively very small and consists of loosely arranged fibroblasts and brown blood pigment cells. Blood vessels are frequent.

The difference between auto- and homeograft is sharp after 13 days. In the homeograft only a few peripheral follicles are present; the height of the epithelial cells varies, with a tendency to become lower. Mitoses are rare, and frequently the epithelial cells are desquamated. Colloid is present only in small amounts; but the lymphocytes, which invade and destroy the follicular epithelium, are frequent. A few polynuclear leucocytes are also present. The follicles are separated from each other by rather broad connective tissue septa, which appear frequently hyaline. These septa completely compress and secondarily destroy the follicles. Lymphocytes are present in large numbers and frequently form large nests. The center is filled with a dense, sometimes hyaline connective tissue, in which elongated nuclei and a small number of lymphocytes are present; the latter also fill the blood vessels. The old transplanted blood vessels are totally obliterated and their wall is thickened.

After 14 Days.—The conditions are analogous to those existing after 13 days.

After 16 Days.—Nearly the entire section of the autograft consists of closely arranged small and large follicles with cuboidal epithelium and light vesicular nuclei, which show only rarely a mitotic figure. Vacuolar colloid fills nearly all the follicles; occasionally one sees embedded in the colloid a desquamated cell or an extremely large, swollen vesicular nucleus, with chromatin near the membrane. Lymphocytes and polynuclear leucocytes are absent. Only in a very small part of the center of the section follicles are absent; where they are absent we find numerous connective tissue cells, which may form a loose fibrillar tissue, containing blood vessels, several foreign body giant-cells, isolated lymphocytes, and numerous brown blood pigment cells.

The two corresponding homeografts differ strongly from the autograft, and also slightly from each other. In one case about four to six partly preserved follicles are present, containing a number of desquamated epithelial cells; numerous lymphocytes surround the follicles, invade the epithelium, and destroy it. Small remnants of colloid are present. The few preserved follicles are separated from each other by broad hyaline connective tissue septa, compressing the follicles, which assume an elongated shape. The larger part of the section con-

sists of a central dense connective tissue with numerous lymphocytes. Far better preserved appears the second homeograft of the same age; here a larger number of follicles with preserved epithelium and colloid is present; the epithelium and nuclei, which still show mitoses, are of the same character as in the autograft, but in the homeograft connective tissue is invading the spaces between the individual follicles, separating them from each other. The invading connective tissue contains a large number of lymphocytes. The arrangement of the follicles is not regular, they are found not only along the periphery, but isolated ones are also seen in the central parts; they are smaller and do not contain any colloid, but even here a mitotic figure is seen in the epithelium. These isolated follicles are compressed by broad connective tissue bands filled with lymphocytes. A number of follicles are destroyed by the invading lymphocytes, but this process of destruction in the second piece is not as marked as in the majority of the homeografts. The homeograft is divided by broad septa into several lobules; a central large hyaline area is lacking. In the central connective tissue mitoses are not infrequent; lymphocytes are numerous, but they are not found accumulated in small clusters. The graft is surrounded by dense connective tissue with lymphocytes, which are also seen in the adjacent tissue together with giant-cells.

After 17 Days.—The autografts show the typical picture of a normal thyroid with closely arranged follicles, lined with epithelium, which surround colloid. The epithelium is high cuboidal, with large, vesicular nuclei, containing rarely a mitosis. Lymphocytes are absent. In a few follicles a slight desquamation, extravasated blood, and blood pigment are found. The small center consists of loosely arranged connective tissue cells, which do not show anywhere the tendency to form a fibrous or hyaline tissue; the larger blood vessels are also located here. The graft is surrounded only partly by dense connective tissue, partly by muscle fibers.

In the corresponding homeograft we observe the results of tissue destruction; almost no follicles are found anywhere, only remnants of destroyed follicles can be seen; they appear as narrow fissures filled with several desquamated cells and lymphocytes. The rest of the graft shows a rather uniform hyaline area, with distinct nuclei; lymphocytes are also present in limited number, located mostly in the periphery, at the place of the former follicles. Several giant-cells are met along the periphery. The larger central blood vessels are totally obliterated and their wall is thickened. The blood supply of the graft is limited; the blood vessels are located mostly in the periphery. The grafts are surrounded partly by a fibrous connective tissue, partly by muscle tissue, containing giant-cells.

After 19 Days.—The regeneration in the autograft is nearly complete. Follicles of various sizes are lying close to each other; the epithelium is somewhat flatter than after 15 and 17 days and the nuclei do not show any mitoses. All follicles are entirely filled with colloid. Lymphocytes are absent. Connective tissue is scant, so that a connective tissue center can hardly be made out. The connective tissue which does exist, however, consists of loosely arranged fibrils with light vesicular nuclei, numerous brownish blood pigment cells, blood vessels, and lymphocytes. Both homeografts show conditions similar to those after 16 days. While in one piece only fibrous connective tissue with several giant-cells

is seen, numerous colloid-filled follicles are still preserved in the other homeograft. The follicles are lined with a cuboidal epithelium, containing as before light vesicular nuclei; the follicles are mostly isolated, surrounded by connective tissue, which in some parts is finely fibrillar, but forms in other places dense fibrous bands, which divide the section into several small parts. This fibrous connective tissue encircles frequently isolated follicles or groups of follicles, compressing them, so that their lumen is represented only by a narrow cleft. The connective tissue is accompanied by numerous lymphocytes, which invade and destroy the epithelium of the follicles. In many places, especially towards the center, the connective tissue septa are broader and the number of follicles between them diminishes steadily; near the center the septa, containing lymphocytes coming from all directions, unite; between them are seen partly destroyed follicles with desquamated cells and elongated spaces, filled with lymphocytes, which probably represent former follicles which have been destroyed. Blood vessels are not numerous, occupying mostly the extreme margin of the graft, which is surrounded by fibrous connective tissue with lymphocytes.

After 22 Days.—The typical picture of the normal thyroid gland is still more pronounced in the autograft; the follicles are larger, especially at the periphery, the epithelium is cuboidal, and the nuclei are light and vesicular, without mitoses. The homeografts, analogous to those after 16 and 19 days, show again a small number of preserved follicles with solid particles of colloid; the preserved follicles are smaller than in the autografts, their epithelium and nuclei are normal, but they are actively attacked by lymphocytes, which are found between the epithelial cells of the follicles, invading and destroying the latter. In general the number of lymphocytes is now larger, while after 16 and 19 days the connective tissue prevailed. But here also the connective tissue encircles the follicles, forming broad septa, which divide the section into several parts; this causes the formation of several small hyaline areas, instead of one large central hyaline field. But altogether the number of follicles injured by lymphocytes is more frequent than the ones compressed by connective tissue. Larger and smaller accumulations of lymphocytes are seen in all parts of the sections. Blood vessels, chiefly situated in the periphery, are, on the whole, rare.

After 26 Days.—Both autografts show again the typical picture of the normal thyroid, consisting of closely arranged, colloid-filled follicles. In the same graft a perfectly normal parathyroid is present, consisting of solid strands of epithelial cells.

After 26 days remnants of the homeografts could not be made out with certainty; the pieces found consist of a dense connective tissue, containing several giant-cells and a few lymphocytes.

After 27 Days.—The picture of the autograft is similar to the autografts 26 days old; perfectly regenerated parathyroid tissue is again present. The homeografts after 27 days are, however, much better preserved than those of the preceding stage. It is surrounded by a dense, moderately broad connective tissue capsule, which sends several septa into the interior of the tissue. The structure of the graft is very dense, as can be seen already under the low power. Along the periphery are found about fifteen to twenty very small round or oval follicles, which are isolated from each other and which still contain solid colloid without vacuoles. In the center only one well preserved follicle is seen. The

epithelium of the preserved follicles is cuboidal with vesicular nuclei. Between the epithelial cells of the follicles lymphocytes are frequently present; they penetrate towards the lumen of the follicles, at the same time injuring the latter. Connective tissue is present in a comparatively small amount; it does not form wide septa which compress the follicles. Lymphocytes, however, are numerous and they accomplish in the main the destruction of the follicles. The blood vessel supply is extremely small in comparison with that of the autograft, where a well developed system of blood vessels is always embedded in the central, loosely built connective tissue, which is entirely absent in the corresponding stages of the homeografts.

After 29 Days.—Both autografts again show the typical structure of the normal thyroid gland. The central connective tissue is very slight, of loose texture, and contains brown blood pigment cells and numerous vessels; lymphatic spaces filled with colloid are also present. The lobular structure of the gland is very distinct; fine septa containing blood vessels traverse the tissue in several directions, without altering the shape of the follicles, or without compressing them. The follicles are very numerous, and of larger size than in the previous stages; their shape is frequently oval or irregular; they are distended with colloid, in which are rarely seen one to two desquamated cells. Well developed follicles filled with colloid are found at several places in the fibrous capsule which surrounds the graft; their shape or size is not changed by the surrounding connective tissue; some show the formation of papillæ. Both homeografts are preserved after 29 days; they show some well preserved follicles, containing colloid. But in both cases a very active destruction of the majority of follicles by lymphocytes and connective tissue is visible, the connective tissue being unequally active in both cases. In the first case wide fibrous septa are passing through the piece, dividing it into isolated lobules, which they surround like a ring; the fibrous tissue invades also the interior of the lobules, surrounding and compressing the individual follicles to such an extent that their total obliteration results. The connective tissue is accompanied everywhere by lymphocytes, which invade and destroy the follicular epithelium; they are found also in large numbers among the desquamated cells. In the second piece a large number of smaller follicles containing colloid is present. Broad connective tissue bands are lacking, but we find long rows of lymphocytes, which penetrate between and into the follicles and destroy them. The blood vessel supply of the homeografts is very scant.

After 31 Days.—The autografts show the same picture as after 29 days. One homeograft shows still a remarkable number of well preserved follicles, filled with colloid, while many of them are attacked by lymphocytes; on the whole, the number of lymphocytes is in this case relatively small and the destructive process is mainly accomplished through compression of the follicles by broad septa of hyaline connective tissue. The follicles are mostly narrow, elongated, and arranged in isolated groups, which are surrounded by a hyaline connective tissue and which become totally obliterated as a result of compression. Some parts of the section appear entirely hyaline; in other parts traces of almost completely destroyed follicles can be recognized; they appear as narrow clefts, filled with desquamated cells and lymphocytes. The homeografts are surrounded by a broad capsule of connective hyaline tissue.

After 33 Days.—We find in the autografts the same conditions as in the previous stages; there is noticeable a very slight desquamation of cells in several follicles. There are no definite traces of the homeografts at this stage.

After 40 Days.—The autografts show markedly larger follicles, especially along the periphery; the epithelium appears flatter, the nuclei smaller; in the larger follicles papillæ are protruding into the lumen. In some follicles a slight desquamation of epithelial cells is observed. A central area of connective tissue can no longer be seen, only near one pole of the piece there is a moderately dense connective tissue with numerous blood vessels, blood pigment cells, several lymphocytes, and a little fat tissue. The graft is surrounded partly by fibrous connective tissue, partly by muscle fibers. In the homeograft we see only fibrous tissue, containing spaces filled with lymphocytes. There are several giant-cells.

After 43 Days.—The autografts from three experiments show again the typical picture of the normal thyroid gland. There is nowhere a compression exerted by the connective tissue. The grafts are surrounded partly by moderately dense connective tissue, containing some lymphocytes; in other places they join directly surrounding muscle tissue; a few follicles may even protrude between the muscle fibers. Some fat tissue and numerous blood vessels are also found at the periphery. No trace of the homeografts could be found after 43 days.

After 47 and 52 Days.—The same conditions are noted; the autografts are of larger dimensions; the tendency of the follicles to protrude into the neighboring muscle tissue is increasing.

After 47 and 52 days no traces of the homeografts were found. We see, therefore, that in this series the homeografts could not be found after the 33d day.

SUMMARY AND CONCLUSIONS.

If we now consider briefly the principal results of our investigation, we can, in comparing the behavior of the thyroid after auto- and homeoplastic transplantation, in general recognize three stages. In the first stage, comprising the first 4 to 5 days after transplantation, there is no noticeable difference between the auto- and homeograft; both corresponding pieces behave in the main alike; large parts of both grafts become necrotic in the center; the necrosis begins shortly after the transplantation and concerns only a part of the periphery; here a narrow zone of thyroid tissue is left; it consists of one to two rows of partly well recognizable follicles. After 48 hours the first fibroblasts, polynuclear leucocytes, and lymphocytes appear in the tissue surrounding the grafts. After 72 hours these elements are present also in the center of the thyroid tissue. At the same date the first mitoses appear in the follicular epithelium; their number is, however, as yet small, but it in-

creases markedly during the following days. After 4 days are found the first newly formed follicles, and from now on their number is increasing. Colloid is present only in small amounts in the old follicles. The central necrotic area still persists, but the masses of detritus are becoming smaller, while the number of fibroblasts and lymphocytes in the necrotic part is increasing; new blood vessels are also visible in the necrotic center.

The second stage, the stage of transition, extends over the next seven days. The earliest, at first slight, differences between the auto- and homeograft appear after 5 days; the difference is usually very definite after 12 days. During this time there is an increase in the number of follicles in the autograft and correspondingly a decrease in the size of the necrotic central area, which is caused by a gradually progressing removal of the necrotic material in the center of the piece. The follicles are lined with a high cylindrical epithelium, which contains numerous mitoses. The number of mitoses increases markedly after the 4th day and reaches a maximum between the 7th and 9th days. Newly produced colloid is always present in the autograft after the 8th day; in the newly formed follicles the colloid contains numerous vacuoles and is almost never retracted, in contradistinction to the solid retracted colloid in the old follicles, which probably was present in the follicles at the time of transplantation. At the end of this period the central part is almost entirely freed from the necrotic masses and is filled with loosely arranged connective tissue cells, between which isolated lymphocytes and polynuclear leucocytes can be found. It contains also numerous blood vessels and brown blood pigment cells.

After 5 days, with the beginning of the second stage, the first as yet rather insignificant differences become noticeable in the homeotransplants. They are as follows: (1) The number of lymphocytes is much larger in the homeo- than in the autograft. (2) The fibroblasts are increased in the central area and these fibroblasts form here very soon firm connective tissue bundles; they surround also the individual follicles or small groups of follicles and form fibrous bands, which are at this stage still very small. During the following days the number of lymphocytes increases and the fibrous connective tissue becomes more prominent, so that 7, 9, 10, and 11 days after

transplantation numerous follicles are not only destroyed by the surrounding and invading lymphocytes, but in addition a part of the follicles is encircled and compressed by wide connective tissue bands. This compression is especially distinct in the central parts of the homeografts. At this stage the absence of a well developed vascular system is already noticeable in the central parts of the homeografts. It is especially noteworthy that the follicles of the homeograft which escaped destruction by the lymphocytes and connective tissue are as well preserved as in the autograft. Colloid is usually present in smaller amounts than in the autografts, even in the well preserved follicles of the homeografts.

It must, however, be stated that some variations occur in this stage in the degree of destruction of the homeotransplanted pieces. Side by side with pieces in which a marked destruction has taken place, there are other pieces which suffered as yet relatively little. But some of the above mentioned differences between the auto- and homeotransplants are always present to a smaller or larger extent. There are also certain variations in the relative strength, with which lymphocytes and connective tissue injure the follicles; in some homeotransplants the attacks on the part of the lymphocytes prevail, while in others those on the part of the connective tissue are more prominent. The homeotransplants, which are relatively little injured at this stage are spared only to be subject to a stronger attack on the part of the lymphocytes and connective tissue during the following stage.

The third stage begins approximately with the 12th day. From now on the difference between auto- and homeograft is sharply defined. In the autografts the regeneration of the thyroid tissue is steadily progressing and is nearly complete after 21 days. After this date the autograft represents throughout the picture of the normal thyroid gland; well developed follicles filled with colloid are surrounded by a very scant connective tissue. Mitoses, which begin already to decrease after the 9th day, are still present at the end of 17 days, but absent after this date. The small amount of loosely built, usually centrally located connective tissue, has no tendency to undergo a fibrous or hyaline change and does nowhere exert a compression on the follicles. It contains always a large number of blood vessels and only a few lymphocytes.

In the homeograft the secondary destruction of the follicles is progressing with great intensity after the 12th day. In contradistinction to a primary degeneration of the follicles, due to the action of substances circulating in the body fluids, which might have been expected to take place, but which in reality does not occur, and which would be followed only secondarily by a connective tissue proliferation and lymphocytic invasion, we may speak of the destruction by means of lymphocytes and connective tissue, which actually does take place, as a secondary destruction of the follicles. This secondary mode of destruction of the follicles through lymphocytes and connective tissue is found exclusively in the homeotransplant. Following this destruction the larger part of the homeografts is occupied by a fibrous, hyaline connective tissue, in which remnants of destroyed follicles in the form of clefts filled with lymphocytes are seen. In several cases the destruction of follicles through lymphocytes, which accumulate in large numbers around the follicles and destroy them very actively, prevails; in other cases the compression of the follicles through fibrous, hyaline connective tissue predominates; in other cases both factors may be about equally active. The few peripheral follicles which escaped destruction are small, probably as a result of compression through the connective tissue; but they may still show normal epithelium and nuclei and may occasionally contain traces of colloid. Newly formed colloid is not present in the homeotransplants at the third stage. It should also be mentioned that the blood supply of the homeografts, especially after the 12th day, is very much restricted and that the blood vessels are mainly located in the connective tissue surrounding the graft, and not in the central connective tissue, which exists here in a much larger quantity and is much denser than in the autograft. At this stage also we find considerable variations in the extent of the injuries, in the individual follicles. But no piece escapes the attack entirely, and those which remain relatively well preserved for a certain period will be attacked at a later date by the connective tissue, as well as by the lymphocytes of the host.

To summarize briefly the principal result: For a short period of time after operation no difference is seen in the behavior of the thyroid after auto- and homeotransplantation. Very soon, however, a

destruction of follicles begins to take place in the homeografts. This destruction is not caused by a direct primary disintegration or solution of follicles, but depends on the destructive activity of (1) the lymphocytes, and (2) of the connective tissue of the host tissue. The former invade the follicles and destroy them directly; the latter grows into the homeografts in larger quantity than into the autografts. In the former it soon becomes fibrous and hyaline; in the latter it remains cellular. The fibrous connective tissue surrounds and compresses and thus destroys the follicles. In some homeografts destruction by means of lymphocytes, in others by connective tissue, preponderates. The rapidity with which the destruction takes place in different homeotransplants also varies. A much better blood vessel supply develops in the autograft than in the homeograft.

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NOTES ON THE SUBCUTANEOUS ABSORPTION AND THE QUANTITATIVE ESTIMATION OF CHOLESTEROL.*

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Cholesterol, now known to be a constituent of practically every tissue of the body, was discovered in gall stones in 1775 by Conradi, and was given its name (cholesterin) by Chevraul in 1815.[1] The pathologist's interest in this substance is aroused by the fact that when isolated chemically it proves to be practically inert, yet it is a constituent of tissues which are continually being broken down and replaced by new tissues, and has manifold functions. What becomes of the cholesterol present in these tissues? Part of it, at least, that is the part which remains after pathological destruction, is found as cholesterol crystals in the necrotic tissues. Thus, in atheroma of the aorta cholesterol crystals are commonly found in abundance, and old hemorrhages or necrotic areas usually contain such crystals. In respect to the absorption of deposits of cholesterol, little is known.

Meyer[2] has described a case of adenomatous cyst of the ovary which ruptured *intra vitam,* causing miliary nodules over the peritoneum, which revealed microscopically giant-cells and cholesterol. Le Count[3] found cholesterol giant-cells in a case of carcinoma of the scalp and a tumor from the scrotum.

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[1] Good general accounts, historical and biological, of the relations of cholesterol are given by Moore, F. C., Cholesterin: Some Account of its Chemical, Physical and Biological Relations, *Med. Chron.,* 1907–8, xlvii, 204; and Glikin, W., Ueber Cholesterine und verwandte Stoffe, *Biochem. Centralbl.,* 1908, vii, 289, 357. McNee, J. W., Cholesterin: An Account of Its Relations to Pathology and Physiology, *Quart. Jour. Med.,* 1913–14, vii, 221.

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[3] Le Count, E. R., Cholesterin Giant-Cells, *Jour. Med. Research,* 1902, vii, 166.

He produced the same histological picture by injecting cholesterol crystals suspended in sterile salt solution into the backs of guinea pigs.

That cholesterol and cholesterol esters of palmitic and oleic acids are resorbed on feeding and are found in the blood in increased amounts was shown indirectly by Pribram,[4] who noted that serum containing cholesterol (but not cholesterol esters) has an antihemolytic effect toward saponin solutions, while normal serum does not. Jankau[5] observed that cholesterol subcutaneously injected disappeared microscopically from the site of injection even in a few hours.

In a rather unsatisfactory chemical experiment, Aguilar[6] found that cholesterol was absorbed when injected subcutaneously into a dog. He injected 0.25 gm. of cholesterol dissolved in 2 c.c. of ether, and eight days later killed the dog, shaved the skin at the site of injection, and removed a piece of skin with the underlying connective tissue until no further traces of cholesterol could be made out. The tissue was analyzed for cholesterol by extracting with ether, the ether extract treated with alcoholic potash to saponify the fats, and after evaporation of the alcohol the cholesterol was extracted from the residue with ether, which was then filtered, the ether evaporated, and from the residue the weight of cholesterol was determined. This proved to be 0.127 gm. The animal, therefore, in a period of eight days had absorbed about half the amount injected. He concludes that cholesterol is absorbed in part, or rather diffuses very slowly about the site of injection.

SUBCUTANEOUS ABSORPTION.

With a reliable method available for the quantitative determination of cholesterol[7] it was hoped that the absorption of cholesterol from the tissues in crystal form could be more satisfactorily demonstrated and followed chemically at definite intervals step by step as it progressed. With this in mind samples of cholesterol were inserted into the subcutaneous tissues of two series of guinea pigs.

Series 1.[8]—Chemically pure cholesterol was placed in a flat tin foil cap (such as is used to cover bottles) and put into a hot air oven at 160° C. until just completely melted, allowed to cool thoroughly, and then, with aseptic precautions, was cut up into small pieces of 0.1 gm. each, accurately weighed.[9] These pieces

[4] Pribram, H., Beitrag zur Kenntniss des Schicksals des Cholesterins und der Cholesterinester im tierischen Organismus, *Biochem. Ztschr.*, 1906, i, 413.

[5] Jankau, L., Über Cholesterin- und Kalkausscheidung mit der Galle, *Arch. f. exper. Path. u. Pharmakol.*, 1892, xxix, 237.

[6] Aguilar, E., Iniezioni di colesterina. Contributo allo studio dell' alimentazione sotto-cutanea, *Boll. d. Soc. di nat. in Napoli* (*1908*), 1909, series 2, ii, 94.

[7] Corper, H. J., A Modification of Ritter's Method for the Quantitative Estimation of Cholesterol, *Jour. Biol. Chem.*, 1912, xii, 197.

[8] Mr. G. T. Caldwell performed some of the cholesterol analyses in this series.

[9] The pieces of cholesterol were about 1 to 2 mm. thick by 4 mm. wide by 8 mm. long. In some cases it was necessary to use two small pieces of cholesterol to obtain the required 0.1 gm.

were then inserted with aseptic precautions subcutaneously into the backs of guinea pigs, and were removed at certain intervals after insertion for chemical analysis and histological examination.

Histological examination revealed nothing of special interest. The pieces of cholesterol are found well invested in a connective tissue capsule which becomes more mature and firm as the time interval after insertion increases. As we are interested only in the final figures obtained at various periods of removal, the results of the chemical analyses will not be given in detail. It is to be noted that the cholesterol in series 1 was competely melted, so that it formed a solid mass of substance, while in series 2 it was just melted sufficiently to hold together and was, therefore, porous.

D.[10] Sample removed 15 dys. after insertion yielded 0.095 gm. of cholesterol.
A. Sample removed 35 dys. after insertion yielded 0.096 gm. of cholesterol.
A. Sample removed 66 dys. after insertion yielded 0.090 gm. of cholesterol.
B. Sample removed 120 dys. after insertion yielded 0.090 gm. of cholesterol.
B. Sample removed 120 dys. after insertion yielded 0.086 gm. of cholesterol.
A. Sample removed 15½ mos. after insertion yielded 0.091 gm. of cholesterol.
A. Sample removed 15½ mos. after insertion yielded 0.092 gm. of cholesterol.
C. Sample removed 27 mos. after insertion yielded 0.084 gm. of cholesterol.
C. Sample removed 27 mos. after insertion yielded 0.079 gm. of cholesterol.
C. Sample removed 27 mos. after insertion yielded 0.084 gm. of cholesterol.

It is noted that in this series the removal of the cholesterol from the site of insertion was slight (16 to 20 per cent.) within a period of twenty-seven months, and even this may be questioned, so that we can consider it a rather slow process when the cholesterol is present in appreciable amount and in compact form.

Series 2.—Here the samples of cholesterol (0.1 gm., accurately weighed) were much more porous than those implanted in series 1; otherwise the conditions of the experiments were the same.

H. Sample removed 16 dys. after insertion yielded 0.096 gm. of cholesterol.
E. Sample removed 20 dys. after insertion yielded 0.090 gm. of cholesterol.
F. Sample removed 9½ mos. after insertion yielded 0.051 gm. of cholesterol.
F. Sample removed 9½ mos. after insertion yielded 0.050 gm. of cholesterol.
G. Sample removed 11 mos. after insertion yielded 0.049 gm. of cholesterol.
G. Sample removed 11 mos. after insertion yielded 0.076 gm. of cholesterol.
G. Sample removed 11 mos. after insertion yielded 0.033 gm. of cholesterol.
E. Sample removed 13 mos. after insertion yielded 0.068 gm. of cholesterol.
E. Sample removed 13 mos. after insertion yielded 0.058 gm. of cholesterol.

As a result of the above experiments it is seen that solid cholesterol is slowly removed from the subcutaneous tissues of the guinea

[10] The capital letters indicate the different guinea pigs; A, B, C, and D (series 1), and E, F, G, and H (series 2).

pig, the rate of absorption being dependent largely upon its physical state of division. It seems probable that the removal of the cholesterol is due rather to absorption than to a mere mechanical action. This removal is probably performed by the fixed tissue cells, of which the giant-cell found around pathological crystals of cholesterol is an expression.

QUANTITATIVE ESTIMATION.

In the hope of simplifying the method previously described[11] for the quantitative estimation of cholesterol and thus also reducing the time necessary for performing the analyses, the following experiments were carried out. Instead of the preliminary extraction of the fats of the tissues to be analyzed for cholesterol before saponification, it was thought possible that direct treatment of the tissues with a large excess of sodium alcoholate on the water bath, the excess to be removed by means of carbon dioxide, would prove sufficient, the rest of the method remaining the same.

Experiment 1.[12]—Two samples of 30 gm. each of rabbit liver (moist weight) were taken; one was directly saponified with 75 c.c. of 5 per cent. sodium alcoholate, and the other was thoroughly extracted with alcohol and ether, and this extract was saponified with 75 c.c. of 5 per cent. sodium alcoholate; from this point both were carried through the method as previously described. The method of direct saponification of the tissue yielded 0.120 gm. of an oily, yellow product, whereas the alcohol-ether extract yielded 0.083 gm. of pale yellow crystals.

Experiment 2.—Duplicate of experiment 1, with 25 gm. of rabbit liver (moist weight). The method of direct saponification of the tissue yielded 0.079 gm. of an oily, yellow product, while the alcohol-ether extract yielded 0.069 gm. of pale yellow crystals.

Experiment 3.—Duplicate of experiments 1 and 2, with 30 gm. of rabbit liver (moist weight), yielded 0.162 gm. of an oily, yellow product by the direct method and 0.108 gm. of pale yellow crystals by the alcohol-ether method.

Experiment 4.—Two samples of 15 gm. each (moist weight) of rabbit brain were taken; one was directly saponified by means of 65 c.c. of 5 per cent. sodium alcoholate, and the alcohol-ether extract of the other by means of 30 c.c. of 5 per cent. sodium alcoholate. The former yielded 0.438 gm. of an oily, brown product, while the latter yielded 0.682 gm. of pale brown crystals.

Experiment 5.—Duplicate of experiment 4, with two samples of 20 gm. each (moist weight) of rabbit brain. The directly saponified tissue (with 75 c.c. of

[11] Corper, H. J., *loc. cit.*

[12] In the following individual experiments the samples of liver, brain, and spleen were not the same, except where stated.

5 per cent. sodium alcoholate) yielded 0.581 gm. of a brownish, slightly crystalline product, and the alcohol-ether extract yielded 0.629 gm. of a crystalline, brown product.

Experiment 6.—Duplicate of experiment 4, with two samples of 5 gm. each (dry weight) of cow brain. The directly saponified tissue, with 75 c.c. of 5 per cent. sodium alcoholate, yielded 0.561 gm. of a brown product, and the alcohol-ether extract, with 50 c.c. of 5 per cent. sodium alcoholate, yielded 0.699 gm. of a crystalline, pale brown product.

Experiment 7.—Duplicate of experiment 4, with 4 samples of 1 gm. each (dry weight) of cow brain. The two samples directly saponified, with 50 c.c. of 5 per cent. sodium alcoholate, yielded 0.110 and 0.047 gm. of product, while the two alcohol-ether extracted products, with 50 c.c. of 5 per cent. sodium alcoholate, yielded 0.106 and 0.108 gm.

Experiment 8.[13]—Two samples of 30 gm. each (moist weight) of steer spleen were taken. One directly saponified, with 75 c.c. of 5 per cent. sodium alcoholate, yielded 0.128 gm. of a crystalline, pale yellow product; and the alcohol-ether extract, saponified by means of 75 c.c. of 5 per cent. sodium alcoholate, yielded 0.150 gm. of pale yellow crystals.

Experiment 9.—Duplicate of experiment 8, with two samples of 10 gm. each (dry weight). The tissue directly saponified with 100 c.c. of 5 per cent. sodium alcoholate yielded 0.197 gm. of pale yellow crystals, while the alcohol-ether extract, saponified with 35 c.c. of 5 per cent. sodium alcoholate, yielded 0.264 gm. of pale yellow crystals.

Experiment 10.—Duplicate of experiment 9, with two samples of 10 gm. each (dry weight). The tissue directly saponified with 50 c.c. of 5 per cent. sodium alcoholate yielded 0.149 gm. of pale yellow crystals, while the alcohol-ether extract, saponified with 35 c.c. of 5 per cent. sodium alcoholate, yielded 0.233 gm. of pale yellow crystals.

Thinking that possibly better results might be obtained by using a strong watery solution of sodium hydroxide directly on the tissue for saponifying, as is used by Pflüger in his method for determining glycogen, and then proceeding with the rest of the method as originally described (passing through the solution carbon dioxide, etc.), we carried out two analyses.

Experiment 11.—Two samples of 10 gm. each (dry weight) of steer spleen were taken, one directly treated with 10 c.c. of water and 50 c.c. of 50 per cent. sodium hydroxide, heated on the water bath for 30 minutes, cooled, carbon dioxide passed to saturation, etc., yielded 0.137 gm. of pale yellow crystals, while the control, carried through the regular alcohol-ether extraction method, yielded 0.250 gm. of pale yellow crystals.

[13] The tissue residues of the spleen in experiments 8, 9, and 10 after alcohol-ether extraction were saponified after addition of absolute alcohol, by means of 100 c.c. of 5 per cent. sodium alcoholate, and then carried through the regular steps of the process for the estimation of cholesterol, but no products were obtained.

Experiment 12.—Two samples of 5 gm. each (dry weight) of steer brain, one saponified with 10 c.c. of water and 50 c.c. of 50 per cent. sodium hydroxide, yielded 0.568 gm. of pale brown crystals, while the control yielded 0.720 gm. of pale brown crystals.

As seen from the above experiments, it is impossible to shorten the procedure for the quantitative estimation of cholesterol, as previously described, by directly saponifying the original tissues, rather than the alcohol-ether extract, by means of a large excess of 5 per cent. sodium alcoholate or by means of a strong solution of sodium hydroxide (50 per cent.).

GENERAL SUMMARY.

Cholesterol, in 0.1 gram amounts, melted in a compact mass and inserted into the subcutaneous tissues of the back of the guinea pig, is slowly removed, as determined by chemical analyses. The rate of removal is probably greatly dependent upon the physical state of the cholesterol.

The quantitative estimation of cholesterol by the method previously described[14] can not be shortened and simplified by the direct saponification of the tissues by means of 5 per cent. sodium alcoholate or a strong (50 per cent.) solution of sodium hydroxide, instead of saponifying the alcohol-ether extract.

[14] Corper, H. J., *loc. cit.*

THE RESISTANCE OF PUPS TO LATE CHLOROFORM POISONING IN ITS RELATION TO LIVER GLYCOGEN.*

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In a former paper[1] it was shown that the various hemorrhagic diseases of the new-born, known as Buhl's disease, Winckel's disease, and melena neonatorum, are probably all expressions of an asphyxial process. This conclusion was based on the following points: (1) the similarity of the anatomical and clinical findings to those known to be induced by lack of oxygen, *viz.*, cyanosis, edema, fat infiltration, hemorrhages, etc.; (2) the experimental production of the various disease pictures by subjecting pregnant animals to the influence of chloroform, a drug which is known to suppress oxidations; (3) the production likewise of similar conditions by direct asphyxiation of the fetuses through ligation of blood vessels to the uterus.

Most of the experiments were made with guinea pigs, but a few dogs were used also.

While the paper was in press, an article by Whipple[2] appeared, in which he showed that in experiments upon four litters of pups he was unable to produce a central necrosis of the liver lobules during the first three weeks of life by the administration of chloroform for a period of approximately two hours. A necrosis of the central portion of the liver lobule is a nearly constant result of a two hour chloroform anesthesia of an adult dog. A portion of the work incorporated in Whipple's article had already been included in one of his former articles.[3] We had, therefore, in our earlier article made note of the absence of findings in Whipple's experiments and had made the suggestion that perhaps a high content of liver glycogen had been concerned in protecting the pups which he used.

* Received for publication, October 31, 1914.

[1] Graham, E. A., The Pathogenesis of the Hemorrhagic Diseases of the New-Born, *Jour. Exper. Med.*, 1912, xv, 307.

[2] Whipple, G. H., Insusceptibility of Pups to Chloroform Poisoning during the First Three Weeks of Life, *Jour. Exper. Med.*, 1912, xv, 259.

[3] Whipple, G. H., and Hurwitz, S. H., Fibrinogen of the Blood as Influenced by the Liver Necrosis of Chloroform Poisoning, *Jour. Exper. Med.*, 1911, xiii, 136.

However, since in our experiments no central liver necrosis was found, although the livers became very fatty, no claim was made that the changes found justified the diagnosis of a true chloroform poisoning, since the presence of a central necrosis has been regarded by some as characteristic of the picture. We felt that perhaps they might be expressions merely of an interference with the fetal blood supply induced by changes in the placenta, of the kind which had already been described by Whipple. Nevertheless, it has seemed desirable to investigate the point further, because from a theoretical standpoint it would be of interest to determine the nature of the relative insusceptibility of pups to late chloroform poisoning. Accordingly, we have carried out experiments with this point in view.

We have been able to corroborate the main conclusion of Whipple that pups are relatively insusceptible to the production of liver necrosis by chloroform. But we feel that the evidence is practically conclusive that this greater resistance is dependent chiefly, if not entirely, on the high glycogen content of the livers of normal, well nourished pups. Our evidence, briefly summarized, is as follows: 1. Pups were made to show the typical liver necrosis, if given chloroform, after adopting measures to deplete glycogen, either by (a) starvation or (b) phlorhizinization and starvation. 2. In adult animals it is well known that increasing the glycogen content of the liver by feeding of carbohydrates serves to protect against poisons which, like chloroform, produce fatty changes in the liver; *e. g.*, phosphorus, arsenic, etc. 3. An examination of the liver of a pup twenty-four hours old showed that as much as 9.07 per cent. of its total weight was glycogen.

It is interesting to compare this amount with the amounts found by Schöndorff[4] in a series of eight adult dogs in which an attempt was made to raise the glycogen content to a maximum by feeding for several days with a large quantity of carbohydrate. The results represent the percentages of the total weights of the livers, and are as follows: 4.3, 7.6, 18.7, 17.1, 16.4, 9.9, 7.3, and 15. Thus it is seen that our finding in the pup's liver of 9.07 per cent. is greater than or equal to the amounts found in half the adult dogs in which an attempt had been made to crowd the liver with glycogen. In other words, this value of 9.07 per cent. may be considered as indicating a liver that is well filled with glycogen.[5]

[4] Schöndorff, B., Ueber den Maximalwerth des Gesammtglykogengehalts von Hunden, *Arch. f. d. ges. Physiol.*, 1903, xcix, 191.

[5] A number of analyses made by Demant (Ueber den Glycogengehalt der Leber neugeborener Hunde, *Ztschr. f. physiol. Chem.*, 1887, xi, 142) on the livers of pups show even higher amounts than the one obtained by us. Thus the livers of three pups, aged 1 hour, 3½ hours, and 3 hours, respectively, showed, by Brücke's method, percentage values of glycogen of 11.389, 9.527, and 5.443, re-

Roger[6] long ago recognized the detoxicating action of the liver against strychnin. Since then it has been learned that the liver exercises a similar action against many other poisonous substances, and that this protective property of the liver is chiefly dependent on glycogen. Rosenfeld[7] has shown that animals fed upon carbohydrates are in general less susceptible to all those drugs which produce fat accumulation in the liver. We have found that generous feeding of sugar to adult dogs renders them much less susceptible to the late poisonous manifestations of chloroform. For a number of years it has been the custom of many of the surgeons in England, where chloroform is extensively used as a surgical anesthetic, to insure in their patients a good supply of glycogen by generous feeding of carbohydrates.[8] Recently, Opie and Alford[9] have shown that in mice the feeding of carbohydrates exerts a decidedly protective action against the development of liver necrosis by chloroform. Furthermore, Rosenbaum[10] has shown that after a protracted chloroform narcosis the liver is actually poor in glycogen.

The mechanism of this protective action of glycogen is not clear. Is it a property of unchanged glycogen itself, or is it rather a property of glucose? It is, of course, well known that in the condition of so called acidosis of the type which occurs during fasting, diabetes, and some other conditions, the feeding of alcohol, sugars, and other substances possessing alcohol groups, is accompanied by a marked diminution in the output of the acetone bodies in the urine. Chloroform poisoning is one of those conditions. The question of how sugars and other substances accomplish a diminution of this

spectively. During the twelve days after birth the amount of liver glycogen was found gradually to diminish. It is interesting to note, therefore, that the diminution of resistance of pups against chloroform poisoning, which occurs with increasing age, parallels a diminution in the amount of liver glycogen. Mendel and Leavenworth (Chemical Studies on Growth.—III. The Occurrence of Glycogen in the Embryo Pig, *Am. Jour. Physiol.*, 1907–08, xx, 117) speak of high values for pups as being in contrast to those for other fetal tissues; and they seriously question the truth of the statement that in general fetal tissue is rich in glycogen.

[6] Roger, G. H., Action du foie sur la strychnine, *Arch. de physiol. norm. et path.*, 1892, iv, 24.

[7] Rosenfeld, G., Fettbildung, *Ergebn. d. Physiol.*, 1903, ii, pt. 1, 50.

[8] Beddard, A. P., A Suggestion for Treatment in Delayed Chloroform Poisoning, *Lancet*, 1908, i, 782.

[9] Opie, E. L., and Alford, L. B., The Influence of Diet on Hepatic Necrosis and Toxicity of Chloroform, *Jour. Am. Med. Assn.*, 1914, lxii, 895.

[10] Rosenbaum, F., Untersuchungen über den Kohlehydratbestand des tierischen Organismus nach Vergiftung mit Arsenik, Phosphor, Strychnin, Morphium, Chloroform, *Arch. f. exper. Path. u. Pharmakol.*, 1882, xv, 450.

particular type of acidosis has been investigated by Woodyatt.[11] On the basis of the so called Cannizarro reaction which has been extensively studied by Ciamician and Silber,[12] he suggests that sugars and other so called antiketogenic substances undergo oxidation with a simultaneous reduction of ketones and keto-acids. But even if this does explain how antiketogenesis is effected, it does not explain the protective action against liver necrosis. We can not seriously consider the necrosis to be dependent upon an acidosis in the ordinary sense; for even in fatal cases of diabetes with high grade acidosis there is no extensive liver necrosis. On the other hand, it is possible that the protective part played by glycogen against chloroform necrosis is more of a physical nature, accomplished perhaps by altering the permeability of the cell or certain parts of the cell to the chloroform. In this connection it has been shown by Bechhold and Ziegler[13] that glucose, alcohol, and glycerin retard the diffusion of some substances into gels. The protective action ascribed by Whipple to the fetal blood islands found in the livers of these young pups has no evidence to support it, except that blood islands are present in the liver of the insusceptible pups; and, moreover, we have found limited areas of necrosis in livers which still contain some of these blood islands, as shown by the presence of an occasional giant-cell and small collections here and there of mononuclear leucocytes. We shall discuss other means of inhibiting the production of this necrosis in another article, now in preparation, in which evidence will be brought to show that chloroform liver necrosis is produced by hydrochloric acid, which is formed as a dissociation product of chloroform, according to the equation:

$$CHCl_3 \rightleftarrows CCl_2 \rightleftarrows + HCl.$$

A number of experimental difficulties were encountered in carrying on the present work. It was found, for example, that it was almost impossible to maintain an even depth of narcosis in the very

[11] Woodyatt, R. T., The Action of Glycol Aldehyd and Glycerin Aldehyd in Diabetes Mellitus and the Nature of Antiketogenesis, *Jour. Am. Med. Assn.*, 1910, lv, 2109.

[12] Ciamician, G., and Silber, P., Chemische Lichtwirkungen, *Ber. d. deutsch. chem. Gesellsch.*, 1901, ii, 1530, 2040; 1902, ii, 1992; iii, 3593; 1903, ii, 1575.

[13] Bechhold, H., and Ziegler, J., Die Beeinflussbarkeit der Diffusion in Gallerten, *Ztschr. f. physikal. Chem.*, 1906, lvi, 105.

young pups; for when the chloroform was given by inhalation they frequently held their breath for periods of from forty seconds to over a minute. Indeed, in one experiment one of these periods of apnea was found to be as long as two minutes and twenty seconds, during which time the pup lay still and appeared to be deeply anesthetized, but in reality it was entirely conscious and reacted vigorously to stimulation by pinching of the skin, etc. To obviate this error regular artificial breathing was usually maintained by alternate compression and relaxation of the thorax. Because of a scarcity of material, the risk of killing pups prematurely by giving the chloroform by injection was not undertaken. Another difficulty was to avoid death from exposure, since in order to shut off the carbohydrate supply, the pups had to be kept away from their mothers. Still another difficulty was in properly gauging the amount of phlorhizin, so that a two hour chloroform anesthesia would not result in the death of the pup during the next few hours. There was no uniformity in tolerance to the phlorhizin, so that this procedure was largely guesswork.

The chloroform used was Mallinckrodt's, bearing the label "Purified for anesthesia." Usually an anesthesia was maintained for two hours, but sometimes for a longer period. After an interval of two days the pups were killed with chloroform and examined. Sections of the liver and other viscera were stained with hematoxylin and eosin, and also with special fat stains, such as Sudan III. After the pups were taken from their mothers they were kept wrapped in cotton, and artificial heat was supplied so that the temperature of the surrounding air in the box was about 30° C. Water was given daily by subcutaneous injection of 20 to 30 cubic centimeters of 0.85 sodium chloride solution. The phlorhizin was injected subcutaneously, sometimes dissolved in warm 1 per cent. sodium carbonate solution, and at other times suspended in olive oil, according to Coolen's method. Typical protocols follow.

STARVATION PRIOR TO ADMINISTRATION OF CHLOROFORM.

Experiment 5.—Mar. 10. Three healthy fox-terrier pups were received into the laboratory, all of the same litter, and five days old. Their respective weights were: A, 237 gm.; B, 275 gm.; C, 350 gm.

Mar. 11. After twenty-four hours of starvation A was given chloroform for two hours, and C for three hours.

Mar. 13. All three pups had lost markedly in weight. The respective weights now were: A, 192 gm.; B, 217 gm.; C, 285 gm. Pup B was given chloroform for two hours. Pups A and C were killed with sudden overwhelming doses of chloroform and autopsied. A shows a moderate amount of fat in the liver with virtual absence of subcutaneous and omental fat. Microscopically the liver shows very marked changes. About the central veins are areas involving roughly one-quarter to one-third of the lobule, in which there is marked necrosis, as indicated by fragmentation of nuclei, hyaline changes in the cells, and deep staining of the necrotic part with eosin. Both here and at the periphery of the lobule the cells are very fatty. Pup C has an excessively fatty liver. There are no important changes in the other organs. Microscopically the liver changes are about the same in kind and degree as those in pup A.

Mar. 14. Pup B was again given chloroform for one hour. At the close of the anesthesia it was cold and in very poor condition; respiration was shallow and infrequent. It was kept all night in an incubator at 35° C.

Mar. 15. Pup B was found in the morning to be in much better condition than in the evening before. Killed with chloroform and examined. Weight, 210 gm. There is marked emaciation with an entire absence of omental and subcutaneous fat; the liver is light brown in color. It is everywhere studded with pinhead-sized areas which are pale and opaque and apparently surround the central veins. Microscopically the liver is found to present marked necrosis and to be intensely fatty. The areas of necrosis are central and involve from one-third to one-half of the liver lobules; they are filled with hyaline areas from which the nuclei have disappeared.

PHLORHIZIN PRIOR TO ANESTHESIA.

Experiment 11.—May 3. Two healthy mongrel pups, two days old, designated as A and B. The respective weights were: A, 390 gm.; B, 370 gm. An injection of 0.05 gm. of phlorhizin was given subcutaneously to A at 9.30 A. M., and repeated at 5 P. M.

May 4. A similar injection of phlorhizin was given again to A at 9.30 A. M. From 1 to 3.30 P. M. both pups were given chloroform. A (phlorhizin pup) took the anesthetic badly and required artificial respiration a number of times. At the close of the anesthesia it was cold and apparently almost dead; both pups were kept in an incubator at about 30° C. all night. We have repeatedly noticed that phlorhizinized animals are much more easily killed with chloroform than are others, a fact which has also been observed by Sansum and Woodyatt[14] in this laboratory. The latter, who made metabolism studies of narcotized diabetic dogs, noted also a great depression of all urinary secretion following the chloroform narcosis.

May 5. B was lively, but A (phlorhizin pup) had shallow respirations and moved only when aroused. At 8.30 P. M. A was moribund; therefore both were killed with chloroform and immediately autopsied. A's (phlorhizin pup) liver was very fatty, B's only moderately so. Microscopically A showed well marked central necrosis involving about one-third of the lobule. B's liver had only a slight amount of necrosis. Both livers contained a few bone marrow cells.

[14] Sansum, W. D., and Woodyatt, R. T., *Jour. Biol. Chem.*, 1915 (in press).

DETERMINATION OF GLYCOGEN.

May 29. Bulldog pup born last night in the laboratory. At 10.30 A. M. it was taken from its mother. At 2.25 P. M. it was killed by decapitation. The liver was immediately removed and ground in a meat grinder. To the hash, which weighed 9.709 gm., were added 10 c.c. of hot 60 per cent. KOH, and the whole was then placed on a steam bath. This and subsequent operations were carried out in accordance with Pflüger's[15] method. After hydrolysis, 0.95 gm. of glucose, which is equivalent to 9.07 per cent. in 9.709 gm. of liver, was found by titration. The unhydrolyzed glycogen was also determined by means of the polariscope. By this method a value of 9.39 per cent. was obtained, which is seen to be in close agreement with that found by titration after hydrolysis.

GLUCOSE AND CHLOROFORM.

Mar. 27. Two small adult dogs, designated as A and B. At 10 A. M. A received 15 gm. of glucose in water by stomach tube. From 11 A. M. to 1 P. M. both dogs were given chloroform. At 1 P. M., while still unconscious, A received 25 gm. of glucose in water by stomach tube.

Mar. 28. A was given 50 gm. of glucose by stomach tube.

Mar. 29. Both dogs were killed with chloroform.

Autopsy.—A's (glucose dog) liver shows a slight amount of infiltrated fat. Microscopically there is no necrosis. B's liver is intensely fatty; and microscopically there is well marked necrosis. The amount of subcutaneous and omental fat was about equal in the two dogs.

This experiment was repeated on two other sets of dogs, and similar results were obtained.

SUMMARY AND CONCLUSIONS.

The relative difficulty with which the characteristic central lobular liver necrosis can be produced in young pups after chloroform administration is in some way referable to the high glycogen contents of their livers. Evidence for this conclusion lies in the following facts:

1. Pups can readily be made to show the central liver necrosis which is found in chloroform poisoning in adults, if, prior to the administration of chloroform, they have been starved or starved and made diabetic by phlorhizin.

2. A single quantitative experiment showed that the liver of a normal, well nourished pup, twenty-four hours old, contained as much as 9.07 per cent. of glycogen.

3. The feeding of carbohydrates to adult animals lessens their susceptibility to the production of liver necrosis by chloroform.

[15] Grube, K., Quantitative Analyse des Glykogens, in Abderhalden, E., Handbuch der biochemischen Arbeitsmethoden, Berlin, 1910, ii, 164.